THE QUANTITATIVE ESTIMATION OF RELATIVE CONCENTRATIONS OF THE VIRUSES OF ORDINARY AND YELLOW TOBACCO MOSAICS AND OF TOMATO SPOTTED WILT BY THE PRIMARY LESION METHOD[1]

by

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INTRODUCTION.

The primary lesion method of estimating relative concentrations of plant viruses as introduced by Holmes (1929a, 1929b) has been further studied by Samuel and Bald (1933) and others for tobacco mosaic virus, by Caldwell (1933) for Aucuba mosaic of tomato and by Best and Samuel (1936) for tomato spotted wilt virus. A good review of the work done on mosaic viruses is given in a paper by Youden, Beale and Guthrie (1935). These latter developed an equation by analogy with the plate method of estimating bacterial populations, which they claimed would apply to all dilution curves. Bald (1935, 1937a) arrived independently at the same conclusion, but considered that only purified virus suspensions gave a curve which agreed with the equation developed. Much of the earlier work on dilution curves was done before it was realized that the salt content and pH value of the inocula had to be controlled to obtain reproducible results.

At various times during the past three years the author has constructed dilution curves with the object of using them to evaluate changes in virus concentration brought about by various chemical treatments. The main conclusions drawn from these curves with respect to the relation between relative virus concentration and the number of lesions have been summarized elsewhere (Best, 1935). Since the form of the curves for tobacco mosaic virus as obtained by the technique used by the author differs from that obtained by other workers, and further since factors hitherto neglected, such as the condition of the inoculated test plants, the time of counting the lesions and environmental conditions prevailing subsequent to inoculation, have been found to affect the estimations for tomato spotted wilt virus, the main results are recorded here. The data show that over a limited but useful range the relationship between virus concentration and the number of lesions is a straight line of unit slope. The work is in no way to be regarded as an exhaustive study of dilution curves *per se* but as a contribution which, it is hoped, will be of use to others in the planning of quantitative work.

[1] Glasshouse facilities and part of the cost of these investigations are being provided by the Council for Scientific and Industrial Research (Australia).

EXPERIMENTAL METHODS.

Methods of raising, selecting and arranging test plants were essentially similar to those described in previous papers. Plants were trimmed and transferred to the laboratory on the morning of the day before they were inoculated. To eliminate differences in plant and leaf susceptibility, test systems were arranged in Latin squares, one square for each leaf level. Also the test systems were inoculated on one half of a leaf only, the other half of every leaf being inoculated with a common control inoculum. Special tests have shown that this practice does not result in any error due to possible contamination. All inoculations of tomato spotted wilt (T.S.W.) virus were made on leaves of *Nicotiana tabacum* (var. Blue Pryor) trimmed to two leaves per plant. Ordinary and yellow tobacco mosaic virus solutions were inoculated on leaves of *Nicotiana glutinosa* plants trimmed to four leaves per plant.

The source of virus, the preparation of inocula and the method of inoculation were as previously described (Best 1936a). The most concentrated suspension (with respect to virus) in each series was used to make up the remaining dilutions in any one experiment. Solutions of ordinary tobacco mosaic virus were prepared sometimes from clarified juice and sometimes from a solution of the purified virus. Yellow tobacco mosaic virus inocula were prepared from clarified juice of infected tobacco plants. Crude juice filtered through fine muslin, was used to make up suspensions of T.S.W. virus.

All suspensions were made in 0·04M composite buffer solution of pH 7. In addition suspensions of T.S.W. virus were made 0·01M with respect to Na_2SO_3 to protect the virus against inactivation during inoculation.

Where only one lesion count is recorded this was the optimal count, i.e. when lesion appearance had almost reached an end point and before coalescence of lesions made counting difficult.

All plants, in any one experiment, were exposed to the same conditions before and after inoculation. Since conditions of light and temperature under which the test plants are raised affect the uniformity of different leaf levels and since the conditions prevailing subsequent to inoculation also affect the form of the dilution curve, a knowledge of these conditions forms an essential part of the data. In order to avoid lengthy descriptions for each experiment, the date of inoculation is given in the tables. This will give a general idea of the conditions prevailing at the time, details of which may be obtained from the Institute records.

EXPERIMENTAL RESULTS.

A. *Ordinary Tobacco Mosaic Virus* (tobacco virus 1).

A dilution experiment, in which clarified infective juice was used as the source of the virus, is summarized in Table 1 and is illustrated in Fig. 1a. A similar experiment in which virus purified by the author's iso-electric precipitation method (Best, 1935, 1936a) was used, is summarized in Table 2 and Fig. 2. In the latter experiment the same spatula was used for inoculating all test systems. To avoid contamination the most dilute solution was inoculated first, followed by the others in order of virus concentration. At the same time the corresponding half leaves were inoculated by means of a second spatula with the common control solution, an aliquot of which was also included as a test system ($\frac{1}{2000}$). In the former experiment the concentration of virus in the control inoculum was such that the number of lesions produced by it approached the limiting value, whereas in the latter one the virus concentration of the control was chosen to fall on that portion of the curve where previous experiments had shown a change in relative virus

concentration to be accompanied by an equivalent change in the number of primary lesions.

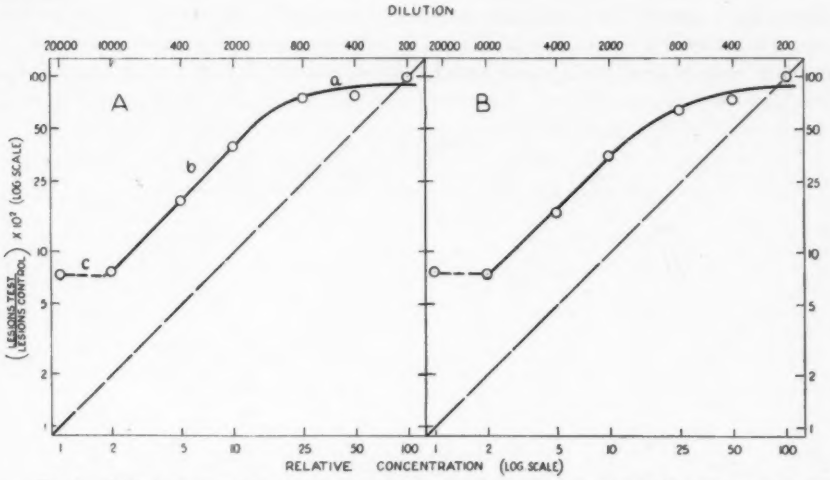

Fig. 1. Dilution curves for A. ordinary tobacco mosaic virus and B. yellow tobacco mosaic virus. In both cases clarified infective juice was used as source of the virus and the concentrations have been expressed as percentages of that of the common control inoculum. All inocula were buffered at pH 7. The line of slope 1 is shown as a broken curve in each case.

TABLE 1.

The relationship between relative concentration of ordinary tobacco mosaic virus and the number of primary lesions produced on *Nicotiana glutinosa* leaves.

[Juice] of control inoculum = 1/200. Temperature of inoculum = 20°C. Date of inoculation 7/4/34. 6 × 6 square of 4-leaved plants.

[Juice] of test	1/400		1/800		1/2,000		1/4,000		1/10,000		1/20,000	
Relative [virus]	0·5		0·25		0·10		0·05		0·02		0·01	
No. of lesions on 24 half leaves	C 988	T 774	C 925	T 700	C 935	T 369	C 894	T 175	C 963	T 74	C 910	T 67
Test / control	0·783		0·757		0·394		0·196		0·077		0·074	

TABLE 2.

The relationship between relative concentration of ordinary tobacco mosaic virus and the number of primary lesions produced on *Nicotiana glutinosa* leaves.

Purified virus was used to make up inocula. A solution of ca. 1·5 mg. virus per litre of 0·04M composite buffer solution of pH 7 was used as the common control inoculum (the equivalent juice conc. of this inoculum was 1/2,000). 8 × 8 square of 3-leaved plants. Temperature of inocula = 18°C. Date of inoculation 17/11/34.

Equivalent [juice] of test	1/1,000		1/2,000		1/3,000		1/4,000		1/5,000		1/7,500		1/10,000		1/20,000	
Relative [virus]	2		1		0·67		0·50		0·40		0·267		0·20		0·10	
No. of lesions on 24 half-leaves	C 211	T 341	C 196	T 189	C 226	T 157	C 220	T 106	C 195	T 85	C 201	T 71	C 231	T 55	C 204	T 32
Test / control	1·62		0·965		0·695		0·482		0·436		0·353		0·238		0·157	

The curve of Fig. 1a may be considered as being composed of three major sections. Section "a" corresponds with high virus concentrations and clearly shows the approach to a limiting number of lesions.[2] Over the concentration range represented by "a" any change in relative concentration of active virus units is accompanied by a much smaller change in the number of lesions. This

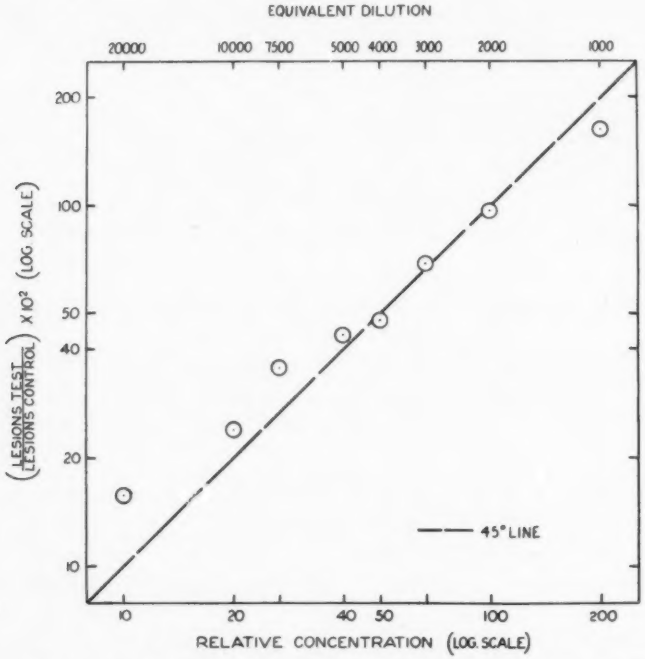

Fig. 2. Dilution curve for ordinary tobacco mosaic virus. Purified virus was used and the concentrations have been expressed as a percentage of that of the common control inoculum. The concentration range with respect to virus was from 3×10^{-6} gm. per ml. to $1{\cdot}5 \times 10^{-7}$ gm. per ml. corresponding with a dilution range (with respect to the volume of juice from which the virus was separated) from 1 in 1,000 to 1 in 20,000. All inocula were buffered at pH 7. The line of slope 1 has been superimposed on the experimental points.

section merges into another, "b", which is essentially a straight line of unit slope. Section "b" covers the concentration range from approximately 1/1,000 to 1/10,000. At still higher dilution a change in relative concentration of active virus is accompanied by a smaller change in the number of lesions, giving the section "c".

[2] The value of this limiting number is determined by the technique of inoculation, the susceptibility of the host plant and the environmental conditions prevailing subsequent to inoculation.

The curve of Fig. 2 covers the concentration range of sections "b" and "c" of Fig. 1a in greater detail. The four points covering the concentration range from 1/2,000 to 1/5,000 deviate from the 45° line by $-3{\cdot}5$ p.c., $+3{\cdot}5$ p.c., -4 p.c. and $+9$ p.c. respectively. These deviations are well within the experimental error. Actually the curve of best fit through all eight points does not deviate significantly from a straight line of slope 1.[3] The points at the lower end of the curve do, however, tend as a whole to swing away from the 45° line to give the beginning of a section comparable with "c" of Fig. 1a.

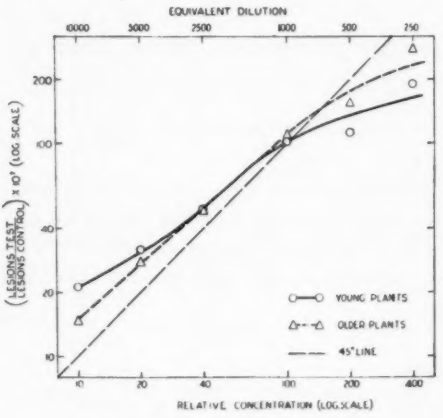

Fig. 3. Dilution curves for ordinary tobacco mosaic virus showing the effect of age of test plants on the form of the curve. Purified virus was used to make up the inocula and the concentrations have been expressed as a percentage of that of the common control inoculum. All inocula were buffered at pH 7.

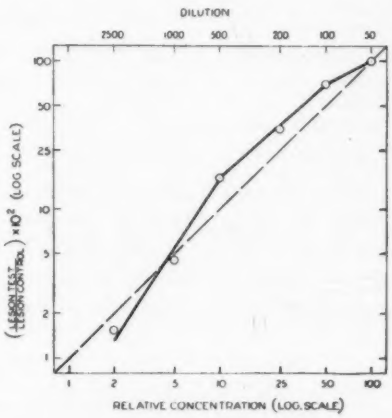

Fig. 5. Dilution curve for tomato spotted wilt virus. Crude infective juice was used as source of the virus and concentrations have been expressed as a percentage of that of the common control inoculum. All inocula were buffered at pH 7 and were 0·01M with respect to Na_2SO_3. The temperature of the inocula when applied was 2°C.

Other experiments suggested that the extent of section "b" of the above curve was affected by the condition of the test plants. To test this an experiment was performed in which two batches of *N. glutinosa* plants, arranged in 6 x 6 Latin squares, were treated with the same inocula at the same time. The plants of both batches were treated alike immediately before, during and after inoculation. They differed only in that one batch was two weeks older than the other. The results (Table 3 and Fig. 3) show that the section "b" was shorter and section "c" was more pronounced for the younger, more susceptible plants.

[3] The author is indebted to Mr. E. A. Cornish for testing this point.

TABLE 3.

Showing the effect of condition of test plants on the form of the dilution curve. Ordinary tobacco mosaic virus (purified) on *Nicotiana glutinosa* leaves.

A. Plants inoculated 6 weeks after germination. B. Plants inoculated 8 weeks after germination. Date of inoculation 16/1/35. Equivalent [juice] of common control = 1/1,000. Temperature of inocula, 21°C.

Equivalent [juice] of test	1/250		1/500		1/1,000		1/2,500		1/5,000		1/10,000	
Relative [virus]	4·0		2·0		1·0		0·40		0·20		0·10	
No. of lesions on 24 half leaves	C	T	C	T	C	T	C	T	C	T	C	T
A. (young plants)	291	555	352	396	270	278	270	134	259	82	303	65
Test / control	1·907		1·128		1·030		0·496		0·316		0·214	
B. (older plants)	45	124	39	61	41	45	62	30	36	10	61	9
Test / control	2·755		1·564		1·098		0·484		0·278		0·148	

B. *Yellow Tobacco Mosaic Virus.*

Table 4 and Fig. 1B summarize a dilution experiment with the virus of yellow tobacco mosaic in which the clarified juice of infected tobacco plants was used as source of the virus. The conditions of the experiment and the procedure were the same as those described for ordinary mosaic (Table 1), and it is evident that the form of the curve is similar in all respects.

TABLE 4.

The relationship between relative concentration of yellow tobacco mosaic virus and the number of primary lesions produced on leaves of *Nicotiana glutinosa*.

[Juice] of control inoculum = 1/200. Temperature of inocula = 17°C. Date of inoculation 21/4/34.

[Juice] of test	1/400		1/800		1/2,000		1/4,000		1/10,000		1/20,000	
Relative [virus]	0·5		0·25		0·1		0·05		0·02		0·01	
No. of lesions on 24 half-leaves	C	T	C	T	C	T	C	T	C	T	C	T
	550	409	468	300	512	181	530	89	439	33	551	43
Test / control	0·744		0·641		0·353		0·168		0·75		0·78	

C. *Tomato Spotted Wilt Virus.*

The data recorded in Table 5A and illustrated in Fig. 4A refer to an experiment in which the test plants were kept in the laboratory during the whole of the period between inoculation and the counting of lesions. Independent Latin squares were arranged, as usual, for upper and lower leaves. This experiment was carried out in collaboration with Mr. G. Samuel, and the curve, previously published without data (Best and Samuel, 1936), was constructed by bulking the data for both sets of leaves. The results for each square are here tabulated and graphed separately.

TABLE 5.

The relationship between relative concentration of the virus of tomato spotted wilt and the number of primary lesions produced by inoculation on leaves of *Nicotiana tabacum*. All inocula were prepared in 0·04M buffer solution of pH 7 + Na_2SO_3 (0·01M).

A. Temperature of inocula = 21°C. [Juice] of common control = 1/30. Date of inoculation 23/3/34.

[Juice] of test	1/15		1/60		1/120		1/300		1/1,500		1/3,000	
Relative [virus]	2		0·5		0·25		0·10		0·02		0·01	
No. of lesions on 6 half-leaves	C	T	C	T	C	T	C	T	C	T	C	T
(a) lower leaves	393	376	402	387	394	322	439	159	389	22	280	10
$\frac{\text{Test}}{\text{control}}$	0·96		0·96		0·82		0·36		0·057		0·036	
(b) upper leaves	699	788	555	558	580	528	724	357	627	43	518	11
$\frac{\text{Test}}{\text{control}}$	1·13		1·00		0·91		0·49		0·069		0·021	

B. Temperature of inocula = 15°C. [Juice] of common control = 1/100. Date of inoculation 28/10/36.

[Juice] of test	1/100		1/200		1/400		1/800		1/1,600		1/2,000	
Relative [virus]	1·0		0·5		0·25		0·125		0·062		0·05	
No. of lesions on 12 half-leaves	C	T	C	T	C	T	C	T	C	T	C	T
	259	348	330	282	232	108	274	46	286	39	290	20
Test[4] / control	1·00		0·64		0·35		0·125		0·10		0·05	

C. Temperature of inocula = 2°C. [Juice] of common control = 1/50. Date of inoculation 2/6/34.

[Juice] of test	1/100		1/200		1/500		1/1,000		1/2,500		1/5,000	
Relative [virus]	0·5		0·25		0·10		0·05		0·02		0·01	
No. of lesions on 12 half-leaves	C	T	C	T	C	T	C	T	C	T	C	T
	549	388	510	180	497	82	550	25	576	9	501	2
Test / control	0·71		0·35		0·165		0·046		0·016		(0·004)	

Table 5A showed that the number of lesions approached a maximum at high virus concentration and that, over the range dealing with the more concentrated inocula, changes in lesion number were proportionately smaller than changes in relative concentration of virus. However, at a juice concentration of about 1 in 100 and for more dilute inocula the change in lesion number was equivalent to the change in relative concentration of virus within the experimental error of the method. This applies to both leaf levels, although there was a tendency for the change in lesion number to be proportionately greater than the change in virus concentration in the experiment on the upper leaves.

In order to determine whether the temperature of the inoculum had any marked effect on the shape of the curve, a dilution experiment was performed in which the dishes containing the inocula were embedded in crushed ice. The temperature of the inocula was about 2° C. The results of this experiment are sum-

[4] Corrected for control spatula.

marized in Table 5C and Fig. 5. The curve is of the same type as those obtained at higher temperatures, so that the temperature of the inoculum itself does not appear to be of importance for this virus.

The concentration of active virus in tomato plants used as source of the virus and the susceptibility of the test tobacco plants vary considerably with environmental conditions, but in general the direct proportionality between lesion number and virus concentration applied only to inocula with a juice concentration equal

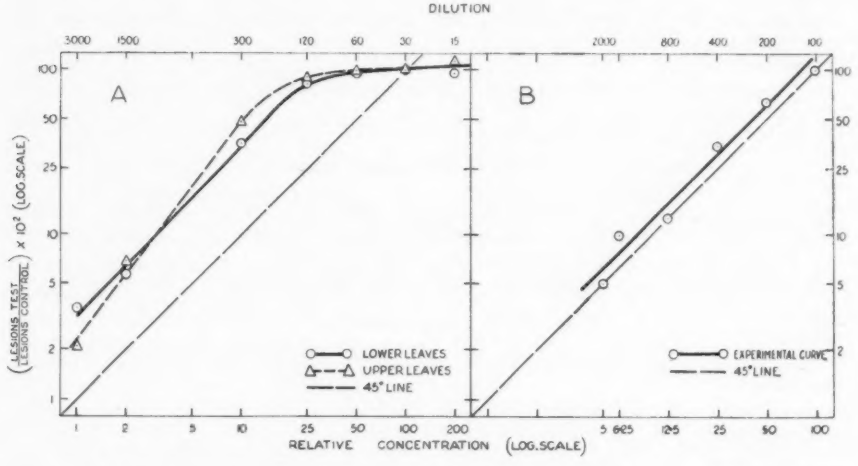

Fig. 4. Dilution curves for tomato spotted wilt virus. Crude infective juice was used as source of the virus and concentrations have been expressed as a percentage of that of the common control inoculum. All inocula were buffered at pH 7 and were 0·01M with respect to Na_2CO_3.

to or less than 1 in 100. This relationship is shown by Fig. 4B constructed from the data of Table 5B. The experiment to which these data relate was carried out in a manner similar to that just described, but in accordance with later practice differences between individual test spatulas were eliminated by a system of rotation. A general correction for the control spatula has also been applied in this case to all the ratios. After inoculation the plants were kept in the laboratory for three days before being transferred to the glasshouse, and the final count was made when lesion appearance had practically reached an end point. The curve is a straight line of unit slope within the experimental error of the method.

At various times dilution experiments have been performed to determine how far this relationship held from time to time as conditions varied, in order that changes in the relative concentration of active virus units brought about by various treatments could be assessed with reasonable accuracy. For the same reason some

other factors, such as the environmental conditions prevailing while the lesions were developing and the time of counting, were also examined. The results of some of these experiments are recorded in Table 6 and Figs. 6A and 6B.

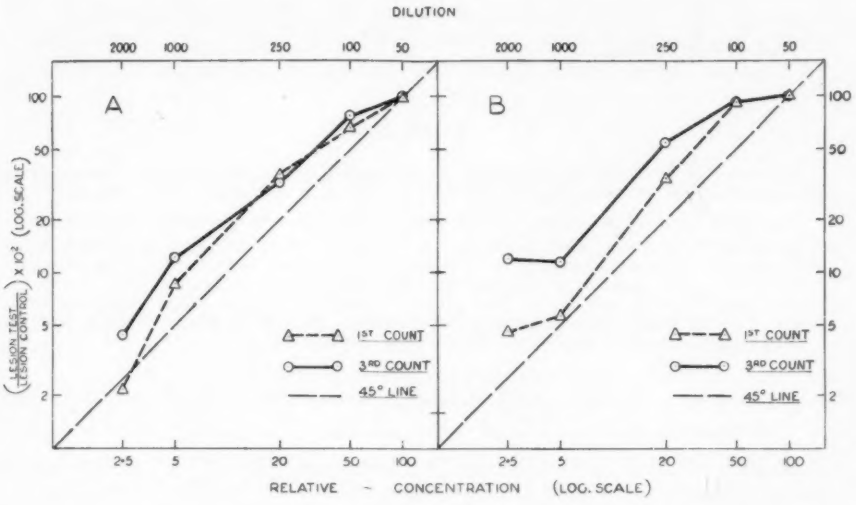

Fig. 6. Dilution curves for tomato spotted wilt virus showing the effect on the form of the curves of time of counting the lesions and also of differences in environmental conditions to which the test plants were exposed after inoculation.

(A) Plants transferred to the glasshouse soon after inoculation.

(B) Plants stored in the laboratory for three days after inoculation and then transferred to the glasshouse.

All plants were inoculated at the same time with the same inocula. Crude infective juice was used as a source of virus and concentrations have been expressed as a percentage of that of the common control inoculum. All inocula were buffered at pH 7 and were 0·01M with respect to Na_2SO_3.

In Fig. 6B the flat portion of the curve at the higher virus concentration is more pronounced and the slope of the middle section (at the first count) is steeper than in 6A. The chief difference between the curves constructed from the figures for the third count (when lesion appearance had nearly reached an end point) is the slope at low virus concentration.

In A the slope is greater than 1 whereas in B it is less than 1. A comparison of the three counts in each group shows that the general tendency is for the ratio $\frac{\text{test}}{\text{control}}$ to become greater with time and that the difference between the ratios at the first and third counts is proportionately greater at the higher dilutions. The tendency for the slope of that portion of the dilution curve which lies below the flat

portion at high virus concentrations, to be greater than 1 is therefore greater during the early counts. It is also greater, the greater the light intensity to which the plants are exposed after inoculation. This lag may receive an explanation along the lines of the discussion of the effect of light on lesion development contained in a previous paper (Best, 1936b, pp. 236, 237). It appears to be due chiefly to an interaction between virus and host plant, but would be accentuated if dilution results in a change in the degree of aggregation of virus particles.

TABLE 6.

The effect of environmental conditions prevailing during the period of lesion development on the dilution curve of tomato spotted wilt virus inoculated on *Nicotiana tabacum* leaves.

A. 16 plants of a 4 × 4 Latin square transferred to the glasshouse 15 minutes after inoculation.

B. As for A., but the inoculated plants were stored in the laboratory for 3 days before being transferred to the glasshouse. The 32 uniform tobacco plants were divided at random into the two groups A and B. Both groups were treated with the same inocula, applied with the same spatulas at the same time. The juice concentration of the common control inoculum was 1/50. All inocula were prepared in buffer solution (0·04M) of pH 7 + Na_2SO_3 (·01M). Temperature of inocula = 17·5°C. Date of inoculation 26/5/34.

		A.								B.							
[Juice] of test		1/100		1/250		1/1,000		1/2,000		1/100		1/250		1/1,000		1/2,000	
Relative [virus]		0·5		0·2		0·05		0·025		0·5		0·2		0·05		0·025	
No. of lesions (8 replicates)		C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
1st count (3 days after inoculation)		170	115	149	54	182	16	138	3	192	178	232	80	229	13	152	7
2nd count (4 days after inoculation)		200	158	193	68	201	24	193	6	312	265	347	171	389	41	237	22
3rd count (5 days after inoculation)		216	169	212	69	202	25	201	9	325	301	353	195	420	48	248	30
Test / control	1st count	0·68		0·36		0·088		0·022		0·93		0·345		0·057		0·046	
	2nd count	0·79		0·35		0·119		0·031		0·85		0·49		0·105		0·093	
	3rd count	0·78		0·325		0·124		0·045		0·925		0·55		0·114		0·121	

Change of Ratio with Time of Counting.

The changing of the value of the ratio $\frac{\text{test}}{\text{control}}$ with time of counting is well illustrated in Table 7 for the viruses of ordinary tobacco mosaic and tomato spotted wilt. The data on which these ratios are based were obtained in experiments carried out in cabinets under controlled light and temperature as described in a previous paper (Best, 1936b) from which the total number of lesions at each count may be obtained. The concentration of inoculum with respect to virus was chosen to fall at the transition point of the dilution curves where the flat portion merges into the straight line of slope 1, and the proportionality between lesion number and virus concentration is known not to hold. This was done in order to determine whether the true ratio is approached at any time.

TABLE 7.

Showing the value of the ratio $\frac{\text{lesions from test}}{\text{lesions from control}}$ at various times after the first appearance of lesions. Virus concentration of the test relative to the control was 0·50 for both viruses.

Tobacco mosaic.			Tomato spotted wilt.		
Time of count in hours after inoculation.	Lesions from test / Lesions from control		Time of count in hours after inoculation.	Lesions from test / Lesions from control	
	15°C.	20°C.		15°C.	20°C.
			65	—	0·44
			71	—	0·44
51	—	0·82	80	—	0·59
61	(0·49)	0·83	90	0·49	0·65
72	0·71	0·83	101	0·51	0·68
82	0·68	0·83	117	0·60	0·71
94	0·69	0·83	125	0·60	—
104	0·71	0·84	138	0·60	0·72
118	0·72	0·84	150	0·64	—
129	0·71	—	162	0·68	0·74
142	0·74	0·85	186	0·71	—
153	0·74	—	210	0·73	—
173	0·74	0·84	235	0·74	—

The ratio did not change appreciably from the earliest to the final counts in the case of mosaic lesions at either temperature, but the value of the ratio was lower at 15° C., being nearer the ratio of the known virus concentrations.

In the case of spotted wilt lesions the ratio had a value, for early counts, near that of the ratio of the virus concentrations of the inocula. The lesion ratio became progressively larger and further removed from the value 0·5. The final ratio was the same at both temperatures and the ratios were comparable at comparable stages of the rate of lesion appearance curve.

The Effects of Leaf Area and Leaf Position.

In all experiments based on a randomized plan, and provided a sufficient number of replicates is taken, the leaf area of all test systems will be approximately the same. The use of a common control inoculum on one half of every leaf and expression of the results as a ratio of the number of lesions on the half leaves of any test system to the number on the corresponding control half leaves, effectively correct for this and other inequalities of the inoculated leaves. However, leaf position (age of leaf) is a far bigger factor than leaf area in determining the number of lesions. When growth is fairly rapid the two contiguous leaves of tobacco plants used for inoculation with T.S.W. virus and the four contiguous leaves of *N. glutinosa* plants (inoculated with tobacco mosaic virus) show little variation in the number of lesions which they will produce with the same inoculum. This state of affairs holds for only a short time, however, and during the greater part of the year no two tobacco leaves (on any one plant) or four *N. glutinosa* leaves will develop comparable numbers of lesions. With tobacco plants sometimes

the older, smaller and sometimes the younger, larger leaves will carry the more lesions. In three typical experiments carried out in autumn, winter and spring, the respective lesion densities expressed as lesions per 100 sq. cm. were 265, 90 and 44 for the lower leaves and 264, 35 and 14 for the corresponding upper leaves. In the last instance the lesion density was thus about three times as great on the smaller, older and less hairy leaves.

The ratio of the number of lesions on the lower leaves to the number on the upper leaves at successive counts during the period of lesion development reached a constant value at an early stage under glasshouse conditions. The ratio also became constant, but at a later stage, in the controlled cabinet experiments.

The possibilities for variation between different leaf levels become greater with *N. glutinosa* plants, where three or four leaves are used. It has been variously reported that the susceptibility gradient (as judged by the number of lesions developed) increases from the bottom leaves upwards or from the top downwards, but Youden and Beale (1934) have shown that this is not always so. This finding has been confirmed by the author's results but it has also been found, as a result of the examination of a large number of experiments (using the half leaves inoculated with the common control suspension as the basis for all comparisons), that only a limited number of the possible combinations occur in practice. It is a matter of general experience that sub-samples of plants taken at intervals from a large batch show at first a rise and then a fall in susceptibility. Very young leaves and very old leaves are less susceptible than leaves at some intermediate stage. It is reasonable to assume that the leaves go through this cycle in the order of their appearance. If we illustrate these cycles of susceptibility by plotting the susceptibility of each leaf against the age of the plant as a series of overlapping curves, then the order of the leaves with respect to susceptibility at any given time will be given by the intercepts with these curves of the perpendicular to the age axis. The possible combinations obtained in this way agree with those obtained in practice.

Susceptibility, as measured by the number of lesions, is a composite thing. It has long been known (Holmes; Samuel) that removal of the growing point some time prior to inoculation reduces the variability between leaf levels, and Bald (1937b) has shown that the addition to an inoculum, of substances which increase its wetting power, reduces the variability between the lesion numbers at various leaf levels for some virus-host combinations. Nevertheless it appears that the internal and external factors which determine susceptibility fit in with the scheme outlined above.

For both virus-host combinations reported here the ratio of the lesions produced by two inocula of different virus concentration often varies with leaf position. This is more pronounced with tomato spotted wilt virus on tobacco leaves. The difference between the ratio for lower and upper leaves is, however, not great and for most purposes the results may be averaged or pooled.

DISCUSSION.

The points of immediate interest have been discussed along with the data in the appropriate sections. The general bearing on the form of the dilution curves of the material presented may be conveniently considered here. Actually each leaf may be expected to have its own dilution curve, but we are unable from the nature of things to determine a curve for an individual leaf. The best we can hope to do is to work with a series of leaves which are as uniform as possible with respect to susceptibility. Dilution curves are constructed on the basis of a large number of leaves at different leaf levels. If the variation between different levels is not large, experience has shown that the results may be pooled. On the other hand, if the variation is large (as for example when a large number of leaves per plant is used) we may approach a state of affairs where all leaf levels do not give the same slope over the same dilution range. It will be readily seen by constructing a series of curves for different leaf levels, how this may give rise to many of the recorded forms. The greater the variation the smaller would we expect the slope of the averaged curve to be. For these reasons the author never uses more than two leaves of a tobacco plant and never more than four leaves of a *N. glutinosa* plant. With these precautions, with control of the inocula in respect to electrolyte concentration and pH value, a controlled inoculation technique and as much control as possible over the conditions under which the lesions develop, the dilution curves are found to have a useful range over which the relationship between virus concentration and lesions produced is a straight line of unit slope within the experimental error of the method.

Little is known about the degree of aggregation of virus particles in the inocula. The equations of Youden and of Bald require that this should not change over the dilution range investigated. The following observations show that it does change in concentrated virus suspensions. If a concentrated solution of tobacco mosaic virus, originally quite clear, is allowed to stand undisturbed for some time it becomes turbid. The virus gradually settles to the bottom of the container as a layer of white (satiny) fibres, leaving a clear supernatant liquid of low infectivity. If the precipitate is distributed uniformly throughout the medium by gentle shaking, the whole becomes opaque, but when in motion still gives the satin-like sheen due to stream double refraction and when a drop is viewed under the microscope the long flexible fibres are found to have been broken up into shorter needles. After vigorous agitation the whole mass becomes translucent to transparent and the virus aggregates are usually no longer visible under the microscope. The whole process is reversible, and ultimately the long flexible fibres are reformed. If a portion containing the undisturbed fibres is diluted to only five times its original volume the threads are no longer visible. The virus aggregates have apparently been broken down to submicroscopic dimensions. This property of the virus molecules which enables them to link up into long chains which dissociate on dilu-

tion may therefore be an important factor in determining the slope of the dilution curve. The chances for a re-aggregation of dissociated particles will become less as the dilution is increased, until at some stage a condition of complete dissociation would be maintained. Just how the degree of association of the virus molecules will affect the dilution curve would depend in part on the size of the minimum or critical infective unit (Best, 1936b). That the susceptibility of the host plants is also a factor which must be taken into account is shown by the data of Table 3 and Fig. 3. These factors may not all be independent variables. In fact so many factors enter into the relationship between concentration of virus and the number of lesions that we could scarcely expect any single equation to represent it over the whole dilution range.

SUMMARY.

The results of dilution experiments with suspensions of three viruses are recorded.

It is shown that in all cases the number of lesions obtained from concentrated inocula approaches a limiting value which depends on the technique of inoculation, the condition of the test plants and the conditions to which the plants are subjected before and after inoculation.

For ordinary and yellow tobacco mosaic viruses the dilution curve may be divided into three portions:

(a) A flat portion at high virus concentrations where a large change in concentration results in a much smaller change in the number of lesions produced.

(b) A straight line section covering a useful concentration range where a change in concentration is accompanied by an equivalent change in the number of lesions.

(c) A section at high dilutions where changes in lesion numbers are again proportionately smaller than changes in concentration.

In the case of ordinary tobacco mosaic virus the shape of the curve is the same whether the inocula are prepared from pure virus or from clarified infective juice.

The general shape of the curve for tomato spotted wilt virus is similar to that of tobacco mosaic except at the lower concentrations where changes in the number of lesions are proportionately greater than changes in virus concentration. This divergence has been shown to depend upon the time at which the lesions are counted and upon the environmental conditions prevailing subsequent to inoculation.

The age and condition of test plants, the time elapsing between inoculation and the lesion count and the conditions prevailing subsequent to inoculation have been shown to affect the form of the curves for one or more of these viruses.

The degree of aggregation of ordinary tobacco mosaic virus in concentrated solutions has been shown to change with dilution, and this and other factors are discussed in their bearing on the form of the curve.

It is concluded that, on account of the number of factors involved, we could scarcely expect a single equation to represent the relationship between lesion number and virus concentration over the whole range. However, in practice under controlled conditions both with respect to inocula and test plants, that portion of the curves over which direct proportionality between lesion number and virus concentration holds, provides a useful working range in which to estimate relative concentrations with a reasonable degree of accuracy.

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THE CIRCULATORY AND PULMONARY EFFECTS OF THE VENOM OF THE AUSTRALIAN COPPERHEAD *(DENISONIA SUPERBA)*

by

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The demonstration that histamine is liberated from the perfused lungs by the venom of the Australian copperhead (Feldberg and Kellaway, 1937), led us to inquire which symptoms of poisoning by the venom may be attributed to this mechanism. It should be possible to gain some information by comparison of the effects of histamine with those of the venom, but in making this comparison the fact that histamine liberated by the venom will produce its main effects at the site of its liberation must be taken into account.

Kellaway and LeMessurier (1936) have already shown that the depressor action of the venom is, like that of histamine, chiefly due to peripheral vasodilatation, though in heart-lung preparations oedema of the lungs and weakening of heart action were observed in the later stages. In the experiments recorded in the present paper we have sought to ascertain whether manifestations of histamine shock, i.e. the conditions of primary and secondary shock, and changes in pulmonary pressure and in blood corpuscle concentration can be brought about by the venom; and, on the other hand, whether histamine can reproduce those lung changes which follow the injection of venom.

METHODS.

The experiments were made on cats anaesthetized with "dial" liquid (Ciba), 0·5 c.c. per kg. being injected intraperitoneally. Both vagi were cut, a tracheal cannula was inserted and the blood-pressure was recorded from the left carotid artery. The pressure in the pulmonary artery was recorded by the method described by Kellaway and LeMessurier (1936). In some experiments the histamine content of the lungs was determined, using the method described in the previous communication (1937). The haemoglobin percentage of the blood was estimated by the Sahli method. Samples of blood were taken with a syringe from the tied femoral artery, which was kept closed with a bulldog clamp in the intervals between the successive samples. The blood in an amount of 0·5 c.c. was mixed with 7 c.c. N/10 HCl. and kept for half an hour before testing. For the estimations we used 0·3 c.c. of this mixture which corresponded to 20 cmm. of blood, the volume usually taken for haemoglobin estimation.

RESULTS.

1. *Primary and Secondary Shock.*

Following the injection of large doses of histamine a primary and a secondary shock have been distinguished. Smith (1928) found in dogs that the arterial blood pressure, which had fallen to a low level immediately after the injection (primary shock), rose again after ten to thirty minutes, gradually reaching a high level, which was maintained for an hour or two, and then fell again slowly to shock

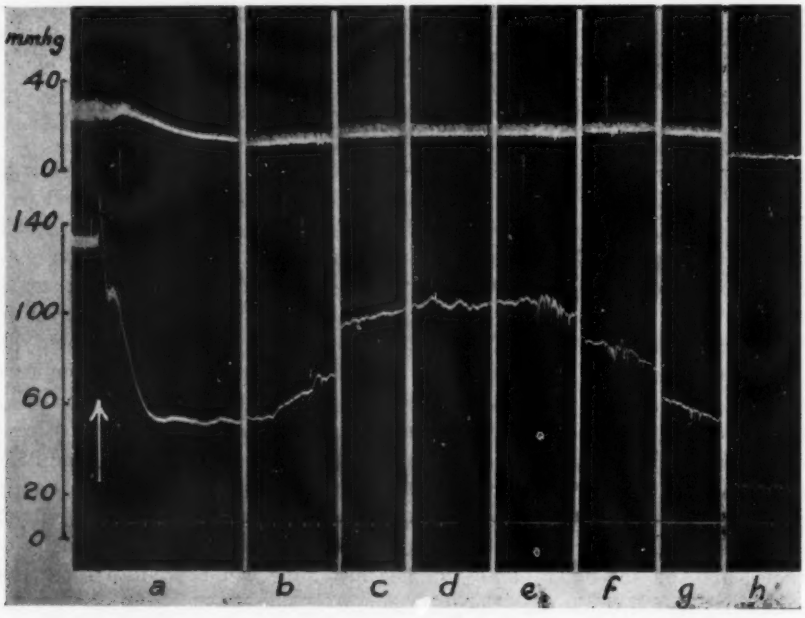

Fig. 1. Pulmonary (upper) and carotid (lower) blood pressure tracings from 2·5 kg. cat under Dial. At the arrow 2 mg. per kg. of copperhead venom intravenously. (b) 15, (c) 25, (d) 30, (e) 35, (f) 45, (g) 50 and (h) 60 minutes after the injection. Time is in half minutes.

level (secondary shock). Feldberg and Kwiatkowski (1933) observed an analogous course of events in cats. They found that secondary shock occurred earlier than in dogs and that the intermediate rise of arterial pressure was a central effect.

A similar phenomenon follows the injection of copperhead venom in suitable dosage. Fig. 1 illustrates such an experiment, in which 2 mg. of venom per kg. were injected. The time relations for primary and secondary shock were the same as those described for histamine. The blood pressure which had fallen immediately after the injection from about 140 mm. Hg. to 55 mm. Hg. gradually rose again

after fifteen minutes (b); it reached a level of about 110 mm. Hg. thirty minutes after the injection (d) and then started to fall again. After sixty minutes (h) the pressure was only 25 mm. of Hg. and the heart action had become feeble and irregular.

With large doses of venom there is generally a steep fall of blood pressure from which no recovery takes place, death occurring a few minutes after the injection. Sometimes the initial fall is interrupted by a transient rise of pressure with acceleration and strengthening of the heart beat. The pressure may even rise above its original level. This rise is probably due to output of adrenaline. Similar events have been described following the intravenous injection of histamine (Burn and Dale, 1926). Small doses of venom cause a transient fall of blood pressure without the later development of secondary shock. The dosage necessary to produce these different effects varies owing to individual variation in the sensitivity of the animals.

2. *The Pressure in the Pulmonary Artery.*

In cats, histamine causes a rise of pressure in the pulmonary artery due to vasoconstriction (Dale and Laidlaw, 1918). With small doses of histamine the rise may be absent and the pulmonary pressure may even fall, particularly if the animal is deeply anaesthetized (MacDowall, 1923). With copperhead venom similar irregularities in the pulmonary pressure can be observed. Kellaway and LeMessurier (1936) found either no effect or a slight fall of pressure in the pulmonary artery after small doses (1 mg. per kg.) of venom. We have confirmed this observation and have often observed a fall in pulmonary pressure even with larger doses of venom (cf. Fig. 1). On the other hand these doses may produce a rise in pulmonary pressure, which accompanies or precedes the fall in systemic blood pressure. Such an experiment is illustrated in Fig. 2. The pressure in the pulmonary artery rose 50 mm. Hg. after the injection. The systemic blood pressure fell steeply to under 20 mm. Hg., no pulsatory oscillations were to be seen on the mercury manometer and the blood pressure record became a thin line. On the other hand vigorous pulsations were maintained in the pulmonary artery and the pressure remained higher than the systemic pressure. If, in such an experiment, the thorax was opened before death occurred, the right heart was found to be distended with blood and beating vigorously. The left heart was small, though it could be felt to be contracting strongly. An incision into it produced very little bleeding whereas if the right heart were incised the thoracic cavity was at once flooded with venous blood. These conditions are characteristic of extreme obstruction in the pulmonary circulation. The fact that little blood was reaching the left heart explains the absence of pulsatory oscillations in the carotid blood pressure despite the actively contracting left ventricle.

In those experiments in which the initial fall in systemic pressure was interrupted, probably by an output of adrenaline, the pulmonary pressure tracing

showed a corresponding notch. In the course of a few minutes the pulmonary pressure usually returned to normal, but during the intermediate rise of systemic pressure, which separated primary and secondary shock, it showed a corresponding slow rise and fall. The slow rise may be due to the development of oedema of the lung and the final fall to failure of the heart.

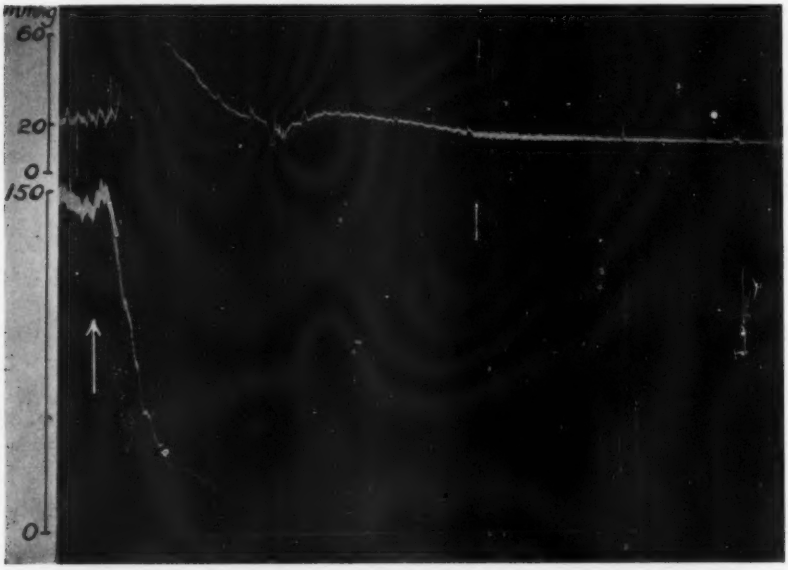

Fig. 2. Pulmonary (upper) and carotid (lower) blood pressure tracings from a 4 kg. cat. At arrow 3·5 mg. per kg. of copperhead venom intravenously. Time is in half minutes.

The initial rise in pulmonary pressure in response to large doses of copperhead venom occurred irregularly. In young and small cats it was generally absent whereas the largest pressor responses were observed in old cats, weighing more than 3 kg. In a recent paper (1937) we have drawn attention to the great variation in the histamine content of the lungs in cats and have pointed out that the lung tissue of young and small cats contains less histamine than that of older and heavier animals. Corresponding observations in other species have been quoted by Feldberg and Schilf (1930). Since histamine is liberated from the lungs by venom, it seemed possible that the sensitivity of the pulmonary vessels to its action might be related to the histamine content of the lung tissue.

In Fig. 3 the histamine content per gram of lung tissue is shown for thirty-five cats. The abscissae give the body weights of the animals in kg. and the ordinates the histamine contents in γ per gram of lung tissue. The data were obtained

from extracts made from portions of the left lung. Generally the histamine content of different portions of both lungs did not differ by more than 20 p.c. so that these figures can be taken as indicating the histamine concentration in the whole lung tissue. In kittens and young cats the histamine content is generally lower than 10γ per gram. In a three days old kitten it amounted to 4·5γ per gram, the

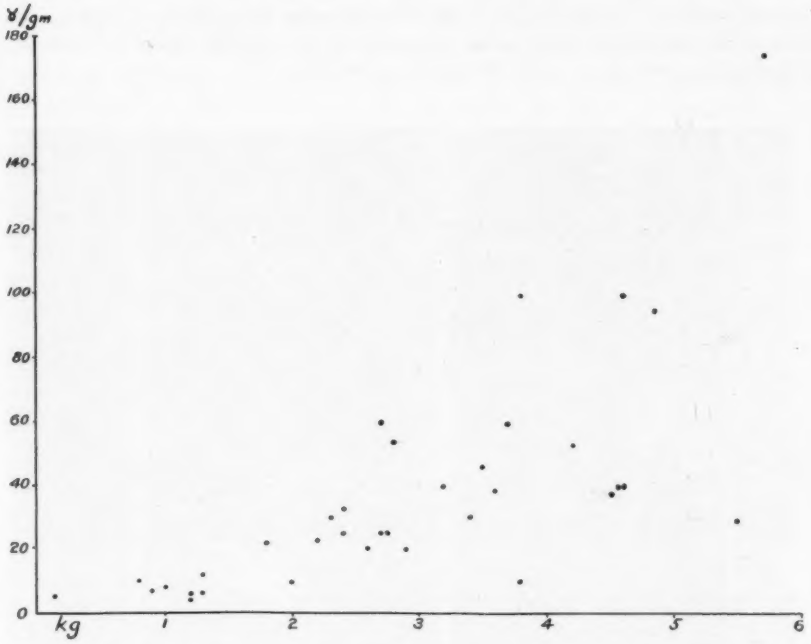

Fig. 3. Histamine content of lung in cats. Ordinates histamine content per gm. lung, abscissae body-weight in kg.

total histamine content of the lungs being 10γ. In cats between 1·5 and 3 kg. weight we found a histamine content ranging between 10 and 60γ per gram but most of the values lay between 20 and 30γ. In cats between 3 and 6 kg. weight we observed variations between 10 and 175γ histamine per gram, and all but two of the values were above 38γ. There was, therefore, a general tendency with increase of body weight to obtain increasing values for the lung histamine. The total histamine content of both lungs in cats weighing over 4 kg. ranged between 700 and 6,700γ. The greatly increased variability in these large animals suggests that factors other than weight and age are operative.

Our results with copperhead venom show a general parallelism between the histamine content of the lung tissue and the response of the pulmonary pressure. No rise, or only a slight rise, was observed in kittens and small cats weighing 1 kg., whereas a pronounced rise was associated with a high histamine content of the lung. Fig. 4 illustrates three experiments in which the histamine content per gram of lung tissue was 10, 25 and 40γ, the pressure in the pulmonary artery rose about 2, 10 and 30 mm. Hg. and the systemic blood pressure fell about 25, 65 and 135 mm. Hg. respectively. In other experiments the depressor effect did not show the same constant relationship to the histamine content of the lungs as the pressor response in the pulmonary artery.

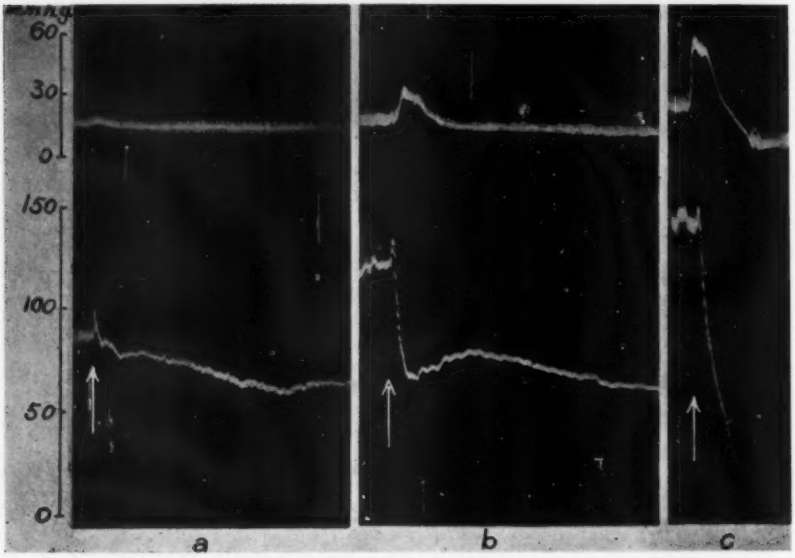

Fig. 4. Pulmonary (upper) and carotid (lower) blood pressure tracings from three cats. At arrow 3·5 mg. per kg. of copperhead venom intravenously. (a) 2·1 kg. cat; 10γ hist. per gm. lung. (b) 2·3 kg. cat; 25γ hist. per gm. lung. (c) 3·8 kg. cat; 40γ hist. per gm. lung. Time is in half minutes.

From these results it appeared that the response of the pulmonary vessels to the venom was *solely* determined by the histamine content of the lung tissue. This, however, was not the case. Experiments, in which the effect of histamine on the pulmonary blood pressure was examined, revealed the fact that the pulmonary vessels of young cats with a low histamine content of the lung tissue were also relatively insensitive to injected histamine, and that the sensitivity increased with increasing content of histamine in the lung. This is illustrated in Fig. 5, which gives

the blood pressure and pulmonary pressure responses in three different experiments. The record (a) was from a 900 gm. kitten, whose lungs had a histamine content of 7γ per gram. The histamine injection produced scarcely any rise of pressure in the pulmonary artery. The fall in systemic pressure was also slight. In other experiments on animals of similar weight and with a similar low histamine content of the lung much greater depressor effects were observed without any pressor response in the pulmonary artery. Similar variations in the sensitivity of the systemic blood pressure of small cats have been observed following the injection of venom. The record (b) was from a 2·4 kg. cat with a histamine content of 25γ per gram and (c) was from a 3·8 kg. cat with a histamine content of 100γ per gram of lung tissue.

3. *Changes in the Lungs.*

We have always removed the left lung before the venom was injected and have used this as a control. It is normally about 15 p.c. lighter than the right lung. If, however, the weight of the small lobe, which is situated distal to the heart is added to that of the left lung the weights of the two sides are in close agreement (see exp. 1–6 of Table 1). This has been done in all experiments in which the two lungs were compared.

TABLE 1.

Effect of Copperhead Venom on Lung Weight of Cats.

Exp. No.	Weight of cat in kg.	Dose of venom in mg. per kg.	Time in minutes between injection and removal of right lung.	Weight of lungs in gm. Left.	Weight of lungs in gm. Right.	Percentage Increase (+) or Decrease (−) in weight of right lung.
1	0·8	—	—	3·7	4·0	+8
2	3·0	—	—	6·9	7·1	+3
3	3·3	—	—	11·6	11·3	−1
4	3·4	—	—	12·4	11·5	−8
5	3·5	—	—	12·2	11·1	−10
6	5·0	—	—	19·1	18·9	−1
7	3·6	2	50	7·0	10·1	+44
8	3·6	2	50	12·1	20·3	+68
9	3·6	2·2	11	17·5	23·7	+36
10	3·0	2·3	45	8·6	12·7	+48
11	3·3	3	11	8·6	15·5	+80
12	1·3	3·5	75	4·9	5·5	+12
13	2·3	3·5	5	7·5	7·7	+3
14	4·8	3·5	5	16·5	16·7	+1
15	2·6	3·5	40	5·7	12·2	+130
16	3·8	5	45	10·5	28·6	+172

The changes produced in the lungs by the injection of large doses of venom differ according to the time of survival of the animals. The lung of an animal which has died within a few minutes following the injection contains little air, though it still floats. It has a dark purple colour and a tough, fleshy consistency.

That there is little or no accumulation of fluid may be deduced from its weight (see exp. 13 and 14 of Table 1). Microscopically the picture is that of atelectasis in a congested lung. An instance of extreme lung collapse is illustrated in Fig. 6 which is taken from experiment 14 of Table 1. Immediately after the injection the

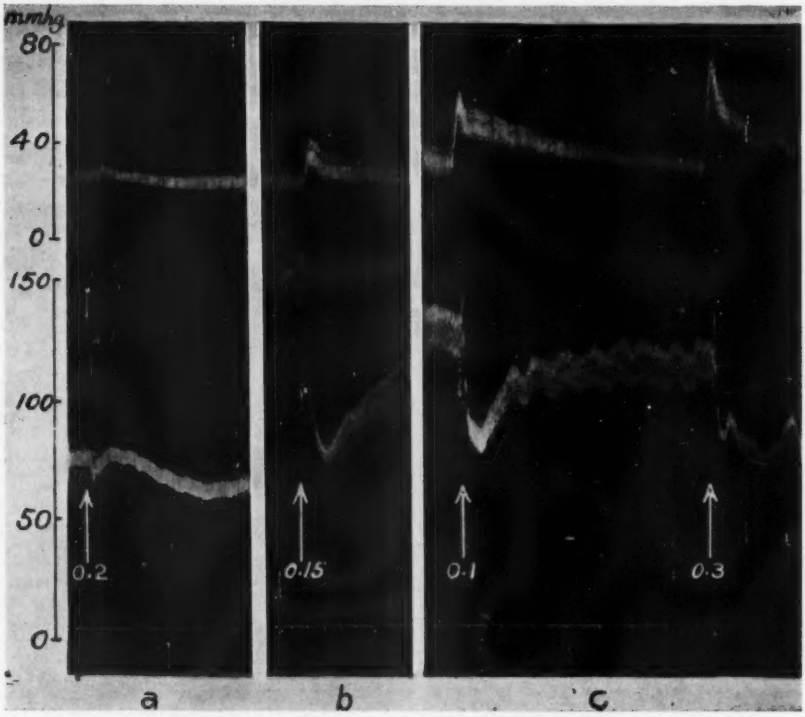

Fig. 5. Pulmonary (upper) and carotid (lower) blood pressure tracings from three cats. (a) 0·9 kg. cat; 7γ histamine per gm. lung. (b) 2·4 kg. cat; 25γ hist. per gm. lung. (c) 3·8 kg. cat; 100γ hist. per gm. lung. The figures give the doses of histamine in mg. per kg. injected intravenously at the arrows. Time is in half minutes.

respiratory movements of the lung, which was being artificially ventilated, decreased; arterialization of the blood became incomplete and death occurred within five minutes. The photomicrograph of a section of the left lung (a) shows much fibrosis. This lung had been removed before the injection and found to contain 95γ of histamine per gram of tissue. In the photomicrograph (b) of a section of the right lung, few alveolar spaces are visible and, owing to the collapse, the fibrosis is more prominent. The sections of the right lung also showed intense congestion,

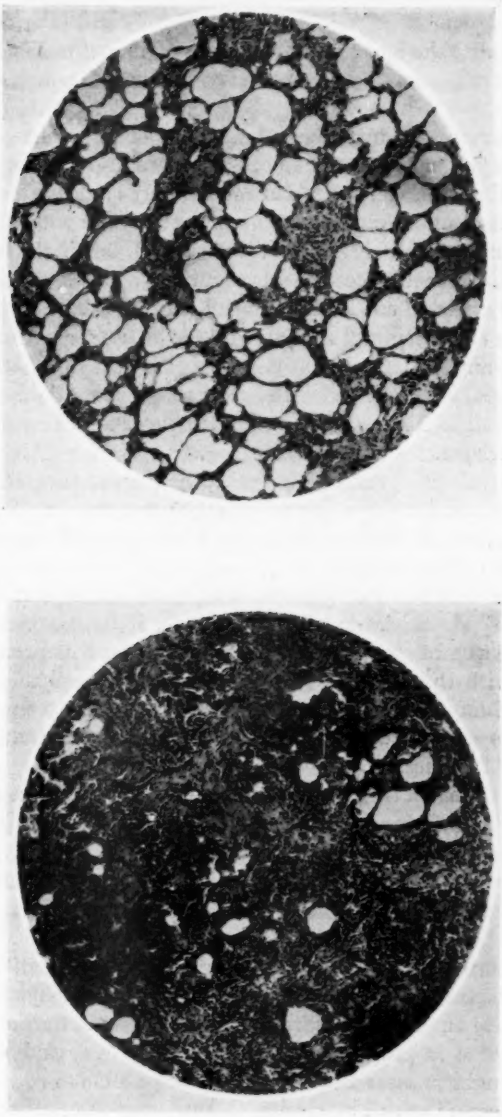

Fig. 6. Photomicrographs of cats' lungs. (a) control left lung; (b) right lung after venom (for details see text).

which cannot readily be demonstrated in the photomicrograph. The collapse, which in this experiment affected the whole of the right lung, accounted for the failure to arterialize the blood and probably contributed to the early fatal result. Such extreme collapse has only been observed in cats with a high histamine content in the lungs.

If the cat survives the injection of large doses of venom for a longer time fluid accumulates in the lung (see exp. 15 and 16 of Table 1). The lung in the open thorax is in a distended condition and relaxes only slightly during the intervals between each inflation. Fluid oozes from the cut surface without frothing, except from the large bronchi. This fluid is blood-stained, but if the lung is allowed to drain for some time the fluid becomes clearer, though it is coloured by haemoglobin set free from haemolyzed corpuscles. Such lungs may show dark purple-coloured areas of collapse which contrast sharply with the bright red colour of the distended portions of the lung. Microscopically in the oedematous portions of the lung the alveoli and bronchi are distended with coagulated oedema fluid, which is also present in the interstitial tissues. The lungs are engorged and there are occasional areas of haemorrhage.

With smaller doses of venom, after which prolonged survival is the rule, similar though not so extensive changes occur in the lungs (see exp. 7 to 11 of Table 1). In kittens the oedema of the lung following a large dose of venom is also less (see exp. 12 of Table 1).

We have observed in the late stages of many experiments some failure to arterialize the blood, which must be the outcome of the extreme oedema. If the blood was removed and shaken up in the air it regained its arterial colour. This occurred also with the poorly arterialized blood from animals which had died within a few minutes after the injection of large doses of venom. In both conditions failure to arterialize the blood must have been due to failure of the respiratory function of the lung.

In animals dying in the course of hours or days after doses of venom in the region of a single lethal dose "starting movements" are sometimes a characteristic feature (Kellaway, 1929). We have observed similar movements in our artificially ventilated, anaesthetized animals in which the blood was insufficiently arterialized owing to failure of pulmonary function. Increase in the artificial ventilation sometimes improved the oxygenation of the blood and at the same time abolished the "starting movements". In other experiments increase in ventilation was ineffective. These "starting movements", which are possibly asphyxial in origin, have been attributed to failure of respiration due to curarization of the diaphragm, but they may be due, in some instances at least, to the lung changes here described.

We have sought to ascertain whether it was possible to reproduce with histamine the lung changes caused by venom. Manwaring, Monaco and Marino (1923) observed in dogs' lungs, which were perfused with solutions containing histamine, changes which resembled those we have described. The lungs became rubber-like

in consistency and there was an "incomplete pulmonary collapse with the escape of large amounts of frothy fluid from the trachea". We found that repeated administration of histamine, in doses between 0·1 and 2·0 mg., either intravenously or into the pulmonary artery caused an immediate decrease of the movements of the artificially ventilated lung, which did not become fully distended during inspiration. If the lung was over-ventilated during that time, it remained distended in the intervals between each inflation. *Post mortem*, the lung was not congested; there were no areas of atelectasis though it was tougher in texture than the normal control lung. Histological sections could not be differentiated from those of the normal control lung. There was usually only slight accumulation of fluid, as indicated by the difference in weight of the lungs of the two sides. In two experiments only the lung weighed 48 and 62 p.c. more than the control lung. These exceptions were lungs with a high histamine content (60 and 100γ per gram respectively). The lung changes after venom were also most pronounced in such animals. We have even observed the onset of spontaneous oedema in an old cat whose lungs contained 90γ of histamine per gram. It seems as though lungs with a high content of histamine are prone to oedema.

The conditions obtaining, following our injection of histamine, are not comparable to those after snake venom in which histamine is being liberated from the cells and acts locally during a prolonged period. The changes described by Manwaring and others following the continuous perfusion of the dog's lung with histamine seem to resemble more closely the effects we observed with venom. However, in previous experiments (1937) in which we perfused guinea-pig's lungs with strong histamine solutions we were not able to reproduce the changes caused by venom. We must therefore attribute, at least in part, the gross alterations in the lungs to direct action of the venom, but we must keep in mind the possibility that the liberated histamine may have more effect on injured than on normal cells.

4. *Fluid Loss from Circulation.*

In 1918 Dale and Laidlaw showed that in histamine shock fluid passes from the circulating blood into the tissue spaces because of increased capillary permeability. This was shown by an increase in the corpuscular content of the blood. Similar changes occur after the injection of copperhead venom.

TABLE 2.

Effect of 2 Mg. Copperhead Venom per Kg. on Haemoglobin Concentration in Cat's Blood.

No. of Exp.	Body-weight in kg.	Haemoglobin p.c. Before venom.	Minutes after venom. 15	25–30	45–50	Maximal Increase p.c.	Calculated fluid loss in c.c.	Notes.
1	3	68	87	95	93	40	43	
2	4	77	107	110	113	47	65	
3	3·6	60	91	85	97	62	69	Left lung removed
4	3·6	69	90	89	87	30	41	before venom injection.

In Table 2 are given the results from four cats, showing a rise of 30–62 p.c. in the haemoglobin concentration after the injection of venom. In the first three the highest values were not obtained until 25 to 45 minutes after the injection, though the arterial blood pressure had in the meantime partially recovered. In another animal, which died three minutes after the injection of 3 mg. of venom per kg., blood removed from the heart at this time showed no increase in haemoglobin concentration. Some time is therefore necessary for the development of the effect.

From the increase in haemoglobin concentration an approximate estimate of the volume of fluid lost from the circulation may be obtained. If we assume that the blood volume of a cat is about 50 c.c. per kg. and that the corpuscles constitute a third of this volume (see Dale and Laidlaw, 1918) then a 3 kg. cat, like that of the first experiment in Table 2, would have 100 c.c. plasma and 50 c.c. corpuscles. A rise of 40 p.c. in the corpuscle concentration, that is from 33·3 to 46·7 p.c., without change in the number of corpuscles would be brought about by a fluid loss of 43 c.c. The fluid loss calculated in the same way for the other three experiments of Table 2 would amount to 65, 69 and 41 c.c. respectively.

The question arose how far lung oedema might account for the fluid loss. In experiment 2 of Table 2 the thorax was opened after the last blood sample had been withdrawn, and the lungs were excised. They weighed 18·6 gm. after 5·4 c.c. of fluid, much of which was blood, had drained from them. The greater part of the 18·6 gm. must be attributed to the weight of the lungs themselves. The fluid accumulated in the lungs could therefore not have accounted for more than 10 to 15 p.c. of the total fluid loss. In order to obtain more accurate data we have compared the weights of the lungs from the two sides, the left being removed before the injection of venom. In experiment 3 of Table 2, for instance, the calculated fluid loss from circulation amounted to 69 c.c., only 3·1 c.c., or less than 5 p.c., of which had accumulated in the right lung. The lung weights of this cat are given in experiment 7 of Table 1. In experiment 4 of Table 2 the fluid loss amounted to 41 c.c. The difference in lung weights (cf. experiment 8 of Table 1) amounted to 8·2 gm.; furthermore 1·2 c.c. of fluid had drained from the right lung before weighing. Assuming that more than half of it was due to oedema fluid, the volume accumulated in the lung would correspond to about 9 c.c. or to 22 p.c. of the total loss. If the left lung had been left *in situ* and had taken up a corresponding amount of fluid, another 9 c.c. would have disappeared from circulation, bringing the fluid loss into the lungs up to 36 p.c. These two experiments show that the oedema of the lungs accounts for a varying proportion but not for the whole of the loss of fluid from circulation. Increased permeability of the capillary walls of the peripheral vessels must therefore be regarded as the main cause of the fluid loss after venom as after histamine.

When samples of blood from envenomed cats were mixed with HCl the mix-

ture changed in colour from red to brown within about five seconds instead of about fifteen seconds as is the case with blood of normal cats. The venom probably rendered the corpuscle membranes less resistant to acid.

DISCUSSION.

The experiments recorded here show the close similarity between the circulatory effects of histamine and those of the venom of the Australian copperhead. In appropriate dosage, both poisons cause an initial precipitous fall of blood pressure, due mainly to peripheral vasodilatation, though pulmonary vasoconstriction may contribute to it. With both poisons this initial fall may be interrupted by changes probably due to the output of adrenaline and, after a variable period during which the blood pressure is maintained at a higher level, a secondary fall occurs terminating in death. A characteristic feature of histamine shock is the increased permeability of the capillaries which allows escape of fluid from circulation, as is evidenced by increase in the corpuscular concentration of the blood. A similar fluid loss occurs after the injection of venom, though lung oedema contributes more to this effect than in histamine shock.

The resemblance in so many details between the effects of histamine and those of venom, coupled with the fact that venom liberates histamine from the perfused lungs, can only mean that the liberation of histamine plays an important part in the symptoms of poisoning with this venom. The study of the changes of pressure in the pulmonary artery seemed to afford conclusive evidence for this conception. We found a parallelism between the sensitivity of the lung vessels to venom and the histamine concentration of the lung tissue. When the latter was low, as in kittens and young cats, the pulmonary vessels were relatively insensitive to venom. It thus appeared as though the amounts of histamine available for liberation were below the threshold of effectiveness. We found, however, that the pulmonary vessels of lungs with a low histamine content were not only relatively insensitive to venom but also to injected histamine, so that the results of these experiments, though they provided further support for our theory, were not decisive.

There was also a parallelism between the histamine content of the lungs and the morbid changes produced by the venom, though these cannot be attributed wholly to liberated histamine since they could not be reproduced by its injection. Congestion, collapse and oedema were more pronounced following the injection of venom in those cats whose lungs contained a large store of histamine. We have made corresponding observations (1937) in experiments on perfused lungs. Thus we see that susceptibility of the lungs to cell injury, and of the lung vessels to histamine and to venom and a high histamine content of the lungs are generally associated. If we can regard the liberation of histamine as part of a defensive response against injurious agents, it is interesting to note that the conditions in which histamine can be liberated in greatest amounts are those in which it is most

effective and in which also the direct effects of cell injury are most pronounced. Age and body-weight have a significant influence in determining this complex association, but the extreme variability observed in old and heavy cats, both in histamine content and in susceptibility to histamine and to venom, must be related to other unknown factors.

We may conclude that the pulmonary and circulatory effects of the venom are due to a combination of the direct results of cell injury and of histamine liberated in the process, though we do not know whether histamine acts only at the site of liberation or escapes also in effective amounts into the circulating blood. It is not surprising that the shock-like condition in snake venom poisoning resembles more closely shock due to anaphylaxis or peptone than that following the injection of histamine, since the first three in varying degrees are complicated by the direct effects of cell injury.

SUMMARY.

To ascertain the part played by liberation of histamine in the symptomatology of poisoning by copperhead venom, the effects of intravenous injection of this venom in cats have been examined and compared with those of histamine.

1. On the arterial blood pressure a condition of primary and of secondary shock can be distinguished after venom.

2. The pressure in the pulmonary artery remains unaffected or falls after small doses of venom; after larger doses it rises in some cats and falls in others.

3. There is a parallelism between the effect of venom on the pulmonary pressure and the histamine content of the lung tissues. The lungs of kittens and young cats have a low histamine content and the pulmonary vessels of these animals are relatively insensitive to venom. The lung histamine increases in older and heavier cats and the sensitivity of the pulmonary vessels to venom is also greater. However, the pulmonary vessels of cats with a low histamine content in their lung tissue are also relatively insensitive to injected histamine.

4. The lungs of old cats, which die within a few minutes after the injection of a large dose of venom, are collapsed and congested; if the survival period is longer, fluid accumulates in the congested lungs which show areas of haemorrhage. The lung changes may lead to failure of the respiratory function of the artificially ventilated lung and to incomplete arterialization of the blood. Injections of histamine, either intravenously or into the pulmonary artery, cause little fluid accumulation in the lungs, which becomes somewhat tougher in consistency due to bronchoconstriction.

5. An increase of 30 to 60 p.c. in the corpuscle concentration of the blood, due to fluid lost from circulation, occurs after the injection of venom. Lung oedema accounts for part only of the fluid loss which is mainly due to increased permeability of the peripheral capillaries.

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THE SALT AND WATER METABOLISM OF NEPHRECTOMISED RABBITS

1. THE EFFECT OF THE INJECTION OF WATER OR GLUCOSE SOLUTIONS.

by

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INTRODUCTION.

In the diuresis produced by the ingestion of sodium chloride the urine ceases to be excreted at an increased rate when only portion of the excess salt has appeared in the urine (Arden, 1934). Further, there is an increase in serum chloride with a return to normal before all the excess salt has been excreted and while the urinary chloride concentration is still high (Arden, 1934; Melli and Tasso, 1930).

Similarly, examples may be cited in which the serum chloride which falls following the ingestion of water returns to normal long before the resulting diuresis has ceased (Dresel and Leitner, 1931). Also, the removal of both kidneys in animals, or anuria in the human subject, causes but little distress for several days. Such observations indicate that extra-renal factors are involved in the maintenance of optimum conditions in the body fluids.

In these experiments an attempt has been made to display these factors, by the use of nephrectomised animals and by the intravenous administration of the test fluids. Thereby it was hoped to eliminate the factors of renal excretion and rate of intestinal absorption, and that, consequently, changes in the blood could be attributed to the extra-renal factors.

EXPERIMENTAL PROCEDURE.

Rabbits of either sex and of approximately 2 kilograms weight were used in this work. The animal was fed on the usual diet of green feed with no additional water, until the first morning of the experiment. Thereafter no food or water was allowed. In the afternoon both kidneys were removed under open ether anaesthesia and the animal left overnight to recover. The following morning the animal was placed in the respiration box (see below) and a sample of blood collected. The test fluid was then injected into the marginal ear vein, and two further samples

collected, one during the first hour and the second two hours or more after the completion of the injection. Thus the blood changes were observed for three hours following the intravenous injection of various fluids into rabbits nephrectomised about 18 hours prior to the experiment.

Injections were made through a blunted serum needle which was inserted into the marginal ear vein. The fluid was led from a burette through a warming bottle to the cannula, so as to be injected at body temperature. The rate of flow was adjusted to complete the injection in from 20 to 25 minutes.

Blood Samples were collected from the marginal ear vein. In some cases considerable difficulty was experienced in obtaining the vasodilatation necessary to produce free bleeding. In such cases, where gentle warming did not produce free bleeding, the attempt was abandoned for 10 minutes and a sample collected as soon as possible. About 3 or 4 c.c. of blood were withdrawn in each sample, so that approximately 1·5 c.c. of blood per kilogram of body weight were lost in this way. Clotting was prevented by the addition of approximately ·01 gm. of potassium oxalate to each tube. Where plasma was required, the bleeding point on the ear was placed under paraffin in a conical vessel containing oxalate. The blood was then centrifuged under paraffin and the plasma withdrawn for analysis.

Recording of Respiration. The apparatus consisted of a box, the lid of which was fitted with sponge rubber edgings so that it could be made airtight. In the front of the box was a round hole through which the head of the rabbit would pass. This hole was covered with sheet rubber in which was a hole of $\frac{3}{4}$-inch diameter. The rabbit was placed in the box, its head forced through the hole in the rubber, and a divided wooden false front with a smaller hole was screwed in place so that the animal could not withdraw its head. The air-tight lid was then clamped in place. The body of the rabbit was thus in an airtight box, the airtight chamber being completed by the snug, but not tight, fit of the rubber around its neck. A side tube led to a loose rubber tambour, the movements of which were recorded on a smoked drum.

METHODS OF ANALYSIS.

Blood solids were estimated by allowing about 0·2 gm. of blood to drop from the ear into a tared crucible, provided with a lid. The sample was immediately weighed and dried at 105°C. for three hours. The sample was then weighed again and the dry weight expressed as a percentage by weight of the original sample. Duplicate estimations agreed with the mean to within 1 p.c.

Sodium in blood or plasma was estimated by the method of Salit (1932). An accuracy of 2 p.c. was obtained.

Chloride was estimated by the method of Smirk (1927), duplicates varying from the mean by less than 1 p.c. In cases where sodium or chlorine was estimated in plasma or serum, the results have been expressed in milligram equivalents per 100 gm. of water. This figure was obtained by determining the water content of 0·1 c.c. samples of plasma by weighing, drying and re-weighing and re-calculating the sodium or chlorine concentrations on the basis of the figure so obtained.

RESULTS.

Controls.

These were animals in which the whole procedure was carried out as above with the exception that no fluid was injected. The animals remained in the box for three hours and samples of blood were collected at intervals during this period.

Blood Solids and Blood Volume. The figures for blood solids are given in Table 1, together with the calculated blood volume. This latter figure was obtained as in the following example (Experiment 24):

Assuming that the initial blood volume was in all cases 55 c.c. per kilogram of body weight (Erlanger, 1921), then total blood solids would be $55 \times \frac{19 \cdot 7}{100}$ $= 10 \cdot 83$ gm. per kilogram, 19·7 p.c. being the blood solid figure found at the beginning of the experiment. At the first bleeding 1·5 c.c. per kilo. body weight were withdrawn, and therefore $1 \cdot 5 \times \frac{19 \cdot 7}{100} = 0 \cdot 29$ gm. solids was lost per kilogram. Thus at the second sampling, when the blood solids were 18·3 p.c., the blood volume would be $(10 \cdot 83 - 0 \cdot 29) \times \frac{100}{18 \cdot 3} = 57 \cdot 7$ c.c. per kilo.

Since it is assumed that 55 c.c. per kilo. is the initial blood volume of all animals, and that the total mass of solids in the blood stream remains unaltered apart from the loss by haemorrhage, and the fact is neglected that blood solid figures are in percentage by weight and not by volume, the blood volume figures must be regarded only as an estimate. Such assumptions are inevitable in any but direct methods of measuring blood volume, but the figure so obtained does take some account of the amount of blood loss, which is not negligible in these experiments.

TABLE 1.

Blood Solids and Calculated Blood Volume in Control Experiments.

No.	Blood Solids, p.c. by Weight.			Blood Volume, c.c. per kg. Body Wt.		
	0	1 hour.	2–3 hours.	0	1 hour.	2–3 hours.
4	13·7	12·7	12·8	55	56·3	52·9
6	19·5	18·6	18·2	55	56·4	56·4
7	18·3	17·0	16·0	55	57·9	60·3
18	16·3	15·3	14·9	55	57·4	57·7
24	19·7	18·3	18·4	55	57·7	55·8
28	17·1	16·3	16·1	55	56·6	56·1
36	18·9	17·8	16·9	55	56·9	58·6
51	21·9	21·6	19·9	55	53·8	56·2
44*	17·6	17·2	17·7	55	56·2	54·3

* In Exp. 44, no blood was taken except approximately 0·2 c.c. per kilo. needed for estimation of blood solids.

It is seen in Table 1 that there is considerable blood dilution in the controls, with an increase in blood volume. In Experiment 44, blood solids only were estimated, so that the blood loss was less than 0·2 c.c. per kilogram, and in this case the blood solid figures showed no significant alteration. Hence it appears that the consistent dilution of blood solids in the control animals is due to haemorrhage, and not to the effects of the nephrectomy. Smirk (1932) has reported similar disproportionate changes in the blood of normal rabbits following small haemorrhage.

Sodium and Chloride in Whole Blood and Plasma. In Table 2 are given the figures for these ions in blood, plasma or serum in the control series. In some cases the figures for sodium and chlorine show increases beyond the limit of experimental error. Possibly more rigorous control of the salt and water content of the diet

would eliminate these variations. For instance, in Experiment 51 where there was a gross increase in plasma sodium, the blood solid figures were very high, which may mean that this animal had become dehydrated as a result of the experimental procedure.

TABLE 2.

Sodium and Chlorine Content of Blood, Serum, and Plasma of Control Animals.

The Figures for Whole Blood are in Mg. Equivs. per 100 c.c. Blood, while those for Serum and Plasma are in Mg. Equivs. per 100 Gram Water.

No.	Fluid Examined.	Sodium. 0	Sodium. 1 hour.	Sodium. 2–3 hours.	Chloride. 0	Chloride. 1 hour.	Chloride. 2–3 hours.
4	Serum	12·5	12·3	12·7			
	Blood				8·0	8·3	8·6
6	Blood				8·2	8·2	8·2
7	Blood				8·5	8·9	9·1
18	Blood				7·2	7·2	7·1
24	Blood				7·3		7·7
28	Blood	8·4	8·2	8·6			
36	Plasma	13·0	13·2	12·8	8·3	8·2	8·4
46	Plasma	12·1	12·4	12·2	8·8	8·9	9·4
51	Plasma	12·7	13·8	14·2	7·8	8·2	8·3

Respiration in the control series showed no regular change in rate or volume. The breathing was frequently irregular, but no gross or persistent changes were observed in any of this series of experiments.

INJECTION OF WATER.

In this series distilled water, 30 c.c. per kilogram of body weight, was injected.

Blood Solids and Blood Volume. The figures (Table 3) show a considerable blood dilution and increase in blood volume. Also during the period of three hours following the injection there was a progressive increase in blood volume; in every case but one, the blood volume at the end of the experiment was greater than that observed soon after the injection.

Blood and Plasma Sodium. The sodium content of whole blood increased greatly following the injection of water (Table 4) presumably due to the increased proportion of plasma (containing sodium) to cells (containing but little sodium) resulting from the blood dilution. If the total sodium in the blood-stream is calculated as the product of the estimated blood volume by the sodium concentration of whole blood (Table 4) and the increase in this figure expressed as a percentage of the increase in blood volume, a surprisingly constant figure is obtained which, moreover, corresponds to the sodium content of plasma. From this it would appear that ultimately the increased fluid in the blood as the result of the injection of water, has the same sodium content as plasma. Accordingly, it was found (Table 5 and Fig. 1a) that the sodium concentration in plasma tended to return to a normal, or even raised, value following the injection of water.

Blood and Plasma Chloride. A similar but rather less constant result is obtained if the figures for chloride are examined in the same way (Table 4). It appears that the plasma chloride does not return to its original value (Table 5 and Fig. 1b).

TABLE 3.

Blood Solids and Blood Volume in Animals Injected with Distilled Water or Glucose Solutions.

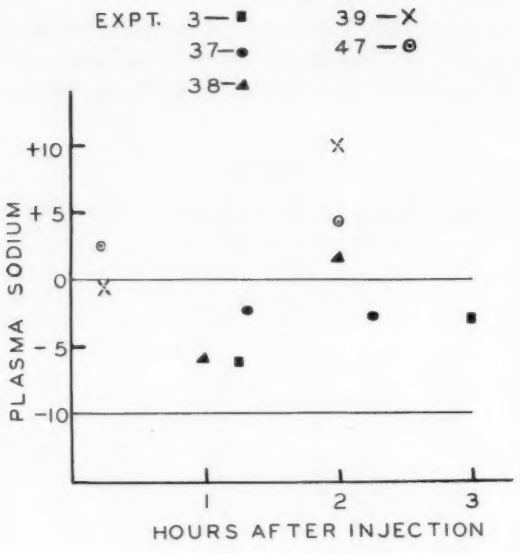
Injection Fluid.	No.	Blood Solids. p.c. by weight.				Blood Volume. c.c. per kg. Body Wt.			
		1	2	3	4	1	2	3	4
Water	3	17·1	—	13·7	12·8	55	—	66·6	69·3
„	5	20·7	—	17·8	16·3	55	—	62·4	66·9
„	8	18·8	—	16·1	15·1	55	—	63·0	66·1
„	15	21·5	19·8	—	17·4	55	58·4	—	65·1
„	16	18·2	15·4	—	13·8	55	63·6	—	69·6
„	21	16·4	14·4	—	12·9	55	61·2	—	67·1
„	22	18·1	15·8	—	14·5	55	61·8	—	66·1
„	29	20·2	—	16·3	14·3	55	—	66·7	74·6
„	30	19·3	16·1	—	15·4	55	64·9	—	66·8
„	32	18·8	15·6	—	13·7	55	64·8	—	72·1
„	33	18·9	—	—	14·1	55	—	—	71·8
„	34	22·6	—	17·9	16·5	55	—	67·9	72·5
„	37	17·3	—	13·5	13·6	55	—	68·2	66·7
„	38	17·9	—	15·2	14·6	55	—	63·0	64·8
„	39	18·9	14·3	—	13·1	55	70·8	—	75·6
Glucose 2 p.c.	43	19·1	—	17·8	16·9	55	—	57·5	58·9
„ 3 p.c.	44	15·9	15·5	—	13·8	55	54·9	—	60·1
„ 3 p.c.	48	20·6	19·3	—	18·4	55	56·6	—	57·2
„ 3 p.c.	50	17·9	16·0	15·8	—	55	59·3	57·7	—

Samples are grouped into four arbitrary time periods:

1. Before injection.
2. From 0 to 15 minutes after injection.
3. From 15 to 75 minutes after injection.
4. More than 2 hours after injection.

Respiration in all these experiments showed a gross and regular alteration. During the injection, or soon after its completion, the respiration became slow, deep and regular—a most profound alteration from the usual fast, shallow and irregular respiration. Actually it was found that the minute respiratory volume was increased about 50 p.c. This alteration reached its maximum within thirty minutes after the completion of the injection and became less obvious as the experiment proceeded; but it usually persisted to the end. The significance of this phenomenon and the associated acid-base changes will be discussed in a subsequent communication.

Haemolysis. When in the later experiments plasma was separated considerable haemolysis was observed, the plasma showing a definite red colour. In order to eliminate the rôle of haemolysis in the above changes, an experiment was performed in which haemolyzed blood was injected in a quantity sufficient to produce



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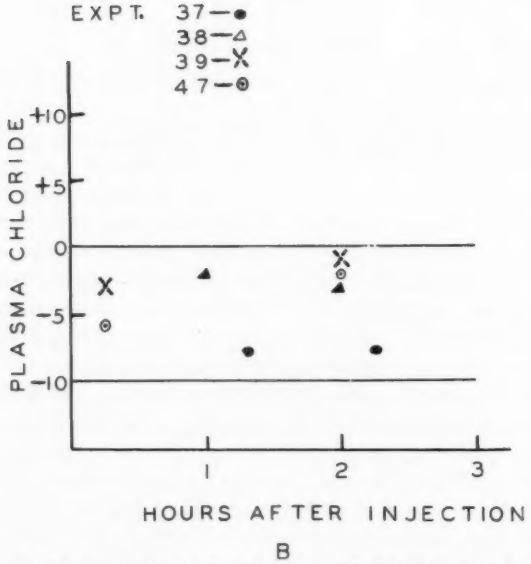


B

Fig. 1. Change in plasma sodium (a) and plasma chloride (b) plotted against time after the injection of distilled water. Sodium and chloride are expressed in percentage increase or decrease of normal.

haemoglobinaemia of approximately the same degree as that observed in the water experiments. The results are given in Table 6. It will be seen that the blood volume figures show no significant difference from the controls. There was, however, a significant rise in the plasma sodium figure which was not present in the chloride estimations. There was no change in the respiration.

TABLE 4.

Blood Sodium and Chloride Figures following Water Injection.

			Sodium.				Chloride.			
No.	Time after Injection.	Blood Volume, c.c/kg.	Blood Concn. mg. equivs./100 c.c.	Total Na in Blood mg. equivs./kg. body wt.	Increase in Total Blood Na mg. equivs./kg.	Increase in Na per 100 c.c. Increase in Blood Volume.	Blood Concn. mg. equivs./100 c.c.	Total Cl in Blood mg. equivs./kg. body wt.	Increase in Total Blood Cl mg. equivs./kg.	Increase in Cl per 100 c.c. Increase in Blood Volume.
	Before	55	7·0	3·87			8·3	4·55		
29	20 mins.	66·7	8·1	5·39	1·42	12	8·4	5·59	1·04	9
	2 hrs.	74·6	8·8	6·55	2·68	*14*	9·0	6·69	2·14	*11*
	Before	55	7·4	4·06			8·1	4·43		
30	3 mins.	64·9	8·2	5·30	1·24	13	7·7	4·98	0·55	6
	2 hrs.	66·8	8·2	5·49	1·43	*12*	8·1	5·41	0·98	*8*
	Before	55	8·0	4·37			7·8	4·29		
32	5 mins.	64·8	8·1	5·27	0·90	9	8·2	5·33	1·04	11
	2 hrs.	72·1	9·1	6·55	2·18	*13*	8·5	6·01	1·72	*10*
	Before	55	7·3	3·99			8·7	4·79		
33	30 mins.		8·3				8·2			
	2 hrs.	71·8	8·3	5·96	1·97	*12*	8·8	6·32	1·53	*9*
	Before	55	6·9	3·80			8·0	4·42		
34	35 mins.	67·9	8·4	5·82	2·02	16	7·2	4·87	0·45	3
	2 hrs.	72·5	8·4	6·11	2·31	*13*	7·7	5·61	1·19	*7*

Insensible Water Loss. Since the deductions from these experiments are based on the assumption that the animal cannot excrete the injected water, several experiments were performed in which the animal was weighed to the nearest gram at intervals during the experiment. In this way it was found that the insensible water loss was small (always less than 1 c.c. per kilo. of body weight per hour). This figure corresponds to that found by Gilman and Barbour (1933), but sufficient accuracy was not attempted to confirm their finding that water ingestion increases the insensible water loss.

INJECTION OF GLUCOSE SOLUTIONS.

To analyse further the effect of haemolysis, experiments were performed in which the same volume (30 c.c. per kilogram) of 3 p.c. glucose was injected. This was found to be the lowest concentration which would prevent haemolysis. It was

found in one experiment that the blood sugar rose from the normal of 0·14 p.c. to 0·33 p.c. fifteen minutes after the injection. After two hours the figure was 0·14 p.c. again.

TABLE 5.

Sodium and Chlorine Concentrations in Plasma Water following the Injection of Distilled Water or Glucose Solutions.

The Figures for Sodium and Chloride are in Mg. Equivs. per 100 Gm. Water.

Injection Fluid.	No.	Sodium.				Chloride.			
		1	2	3	4	1	2	3	4
Water	3*	14·2	—	13·4	13·9				
"	37	13·4	—	13·1	13·0	10·6	—	9·8	9·8
"	38	14·2	—	13·4	14·4	10·1	—	9·9	9·8
"	39	13·4	13·3	—	14·7	10·0	9·7	—	9·9
"	47	12·2	—	12·5	12·7	9·1	—	8·6	8·9
2 p.c. Glucose	43	13·3	—	12·2	13·4	10·0	—	8·9	9·7
3 p.c. Glucose	45	13·1	12·7	—	12·7	9·4	9·2	—	10·0
"	48	12·8	12·1	—	13·0	10·3	9·6	—	9·8
"	50	12·3	12·3	12·4	—	9·0	8·8	8·9	—

* Serum Sodium.

Samples are grouped into four arbitrary time periods:

1. Before injection.
2. From 0 to 15 minutes after injection.
3. From 15 to 75 minutes after injection.
4. More than 2 hours after injection.

Blood Solids and Blood Volume figures are given in Table 3, where it appears that there is less blood dilution than in the corresponding water experiments. From the results of the injection of haemolysed blood, it seems that this difference in the two series cannot be attributed to the effects of haemolysis. Also in experiment 43, a 2 p.c. glucose solution failed to prevent haemolysis, but nevertheless the blood solid figures are typical of the glucose series (Table 3).

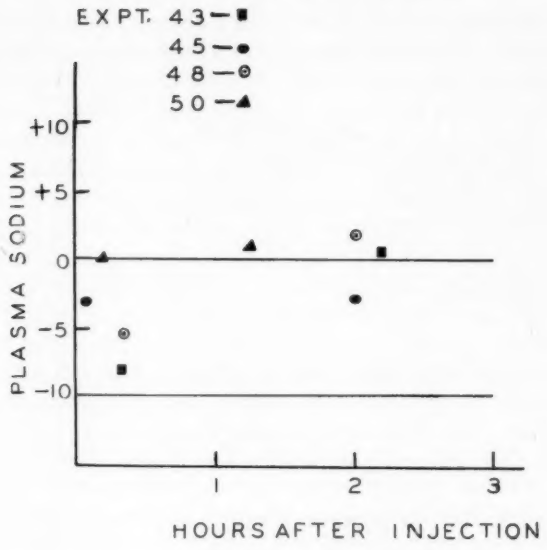
It is also important to note that the blood volume figures show the same progressive increase during the three hours following the injection as that observed in the water experiments.

TABLE 6.

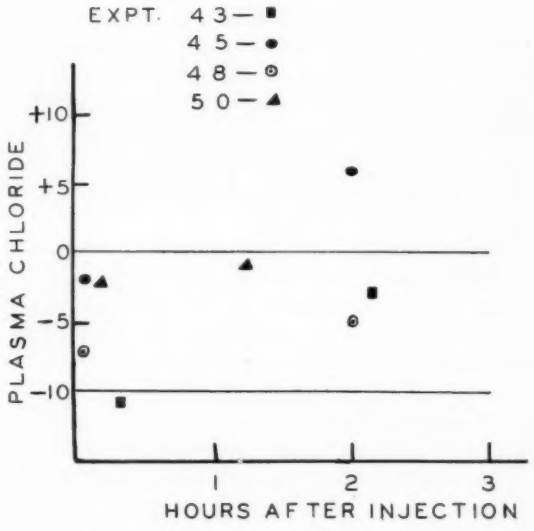
The Effects of Injection of Haemoglobin.

	Before injection.	15 mins. after injection	2 hours after injection.
Blood Solids, p.c. by weight	16·3	15·8	15·3
Blood Volume, c.c./kilo.	55	54·7	54·4
Sodium in Plasma, milli. equivs./100 gm. H_2O	12·6	12·4	13·5
Chloride in Plasma, milli. equivs./100 gm. H_2O	9·3	9·1	9·2

Plasma Sodium and Chloride show the same tendency to return to normal values as in the water experiments. The results suggest that this restoration is effected with greater accuracy in this case (Table 5 and Fig. 2).



A



B

Fig. 2. Change in plasma sodium (a) and plasma chloride (b) plotted against time after the injection of glucose solutions. Sodium and chloride are expressed in percentage increase or decrease.

Respiratory Changes of the same nature as those observed in the water experiments, occurred in this series. There was no difference to be observed in the responses in the two series.

DISCUSSION.

The most satisfactory picture of the internal water metabolism is that given by Peters (1935). Briefly, his view is that blood, interstitial (extracellular) and intracellular fluids constitute the main bulk of the body water. The composition of blood plasma can be determined by direct analysis and, although extracellular fluid cannot be sampled, there is adequate reason to suppose that it is an ultrafiltrate of plasma and, in consequence, its composition may be predicted from plasma estimations. Based on work by Harrison, Darrow and Yannet (1936) and Hastings and Eichelberger (1937) the cells are believed to contain no chlorine or sodium, and consequently the osmotic relationships of extracellular fluid with cells are almost entirely dependent on these ions.

In both of these series of experiments there is an increase in blood volume; in the case of distilled water this amounts to approximately 40 p.c. of the fluid injected. Moreover, the dilution of the blood will cause a tendency for an increased filtration across the capillary walls with a consequent increase in the volume of interstitial fluid. The experiments do not bear on this question of the site of the injected fluid, but from the consideration above it appears that the total extracellular fluid (i.e. blood and interstitial fluid) must be increased. When glucose was added to the injection fluid, the increase in blood volume was far smaller, but the above argument still justifies the belief that the total extracellular fluid is increased. Whether the difference in the two series in this respect represents merely a different partition of fluid between blood and interstitial tissue, or whether the processes of glucose metabolism necessitate the transference of water to the tissue cells, is not clear from this work. The latter explanation receives some support from the work of Gamble, Ross and Tisdall (1933) on the base metabolism in starvation.

In the period of two hours following the injection, the blood volume in both series increases, which indicates that in these experiments there is no evidence that the nephrectomised rabbit has power to dispose of excess water. Consequently, the restoration of plasma sodium and chloride figures towards their normal values indicates that these ions can enter the blood stream and interstitial fluid from some storage area. The rise in salt content of the plasma in control experiments obviously does not detract from the validity of this reasoning. Gollwitzer-Meier and Rabl (1926), from studies of the blood changes following water ingestion by the human subject, also concluded that inorganic ions entered the blood stream as the result of water absorption. Studies from this angle of the parenteral administration of salt-free fluids, so far as I am aware, have not been reported.

From these experiments it appears that dilution of the blood and interstitial

fluid in relation to sodium and chloride content is an adequate stimulation for this influx of these ions into the circulation. This conclusion is not, however, inevitable. It appears, for instance, that changes in plasma composition may occur in control animals, or haemolysis may cause an increase in plasma sodium. Also the hyperpnoea of these experiments suggests that disturbances of the ionic ratios may be a factor in the entry of the sodium and chloride ions into the blood stream. This aspect of the question is at present under investigation.

SUMMARY.

1. A method has been established in which nephrectomised rabbits are used for the study of extra-renal factors in the metabolism of water and inorganic ions.

2. The changes in blood solid content, plasma sodium and plasma chloride following the injection of salt-free fluids (water and glucose solutions) have been studied. It was found that plasma sodium and chloride tend to return to normal values, whereas there is a persistent dilution of the blood in respect to its solid content.

3. The conclusion is drawn that the nephrectomised animal is unable to regulate its blood volume, but that there is a storage of sodium and chloride from which these ions can be obtained to restore normal ionic concentrations in extracellular fluids.

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THE KINETICS OF FORMALIN DISINFECTION OF VACCINIA VIRUS[1]

by

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(Submitted for publication 20th April, 1937.)

The primary purpose of the experiments here reported was to compare the inactivation of vaccinia by immune serum and by formaldehyde, in connection with detailed studies in progress on the method of action of antiviral sera. The findings with formalin have some intrinsic interest, since the pock-counting technique on the chorioallantoic membrane (Burnet, 1936) resembles the plating methods used in the study of bacterial disinfection, and allows more precise and detailed investigation than is practicable with animal inoculation titrations. The kinetics of disinfection by formalin are shown to be the same for vaccinia as for bacteria.

METHODS.

The virus used was the egg passage strain of neurovaccinia (Keogh, 1936). Over 100 passages in the egg have resulted in no apparent change in the characteristics of the lesions on the chorioallantoic membrane. There has probably been some diminution in pathogenicity for the rabbit, shown by failure of generalization following intradermal inoculation. The virus suspensions were prepared by grinding the membrane from an egg, inoculated 2 to 4 days previously, with powdered quartz and emulsifying in 5 c.c. of normal saline containing calcium in Ringer proportions and 10 p.c. normal horse serum (pH 7·5). The required dilutions of virus were made in the same fluid. Although the presence of protein complicated some experiments, owing to absorption of formalin, this diluent has the advantage of minimizing non-specific inactivation of the virus. Further, since the virus suspensions always contain protein of non-viral origin, it seemed preferable to avoid such large differences in protein concentration in varying virus dilutions as would be introduced by diluting in a protein-free diluent.

The formalin dilutions were prepared from commercial formalin (38·1 p.c. formaldehyde by titration) in normal saline. Equal volumes (usually 1 c.c.) of virus and formalin dilutions were mixed. At appropriate intervals the reaction was stopped by dilution 1: 100 or 1: 1,000 in the described diluent. The experiments were carried out at room temperature except when the contrary is stated.

The virus in the samples was titrated on the chorioallantoic membrane by the method previously described (Keogh, 1936). Usually five eggs, sometimes four, were used per dilution. The probable error of the mean pock count of five membranes is of the order $\pm$ 15 p.c. It is assumed that the pock count is directly proportional to the number of viable virus particles in the inoculum. The terms "surviving particles" and "reduction in titre" are based on this assumption.

[1] This work was carried out under a grant for work on virus diseases from the Commonwealth Government Department of Health and the Rockefeller Foundation.

[2] Research Officer, Commonwealth Serum Laboratories.

As most of the experiments were carried out with mixtures containing 0·05 p.c. formalin, this has been taken as unit concentration for the purpose of calculation of constants.

RESULTS.

(a) *Virus Concentration.*

Within the experimental range and under constant conditions of time and temperature, the percentage reduction in the number of viable virus particles effected by a given concentration of formalin is independent of the virus concentration.

TABLE 1.

The "Percentage Law".

Virus dilution.	Formalin concentration.	Average lesions per 0·05 c.c. of 1: 100 dilution of mixture.	Percentage survivors.
1: 10	0·1 p.c.	55	8·5
1: 20	"	31	9·5
1: 40	"	17	10·5
1: 80	"	7	8·6
1: 160	"	4	9·9

Control virus dilution averaged 13 lesions per 0·05 c.c. of 1: 10^5 dilution.

The "percentage law" of Andrewes and Elford (1933), shown by them to hold for the inactivation of bacteriophages by immune sera and by formalin, and which also applies to the inactivation of several viruses by immune sera (Burnet, 1936, Keogh, 1936) holds, therefore, for the inactivation of vaccinia by formalin.

(b) *The Effect of Time.*

The inactivation progresses steadily with time. If the logarithms of the numbers of surviving particles are plotted against the time of disinfection, the points lie approximately on a straight line, within the range of experimental error. The relation can be expressed $kt = \log. \frac{V}{v}$ where V = the original number of virus particles, and v = the number of particles surviving disinfection. Table 2 records a typical experiment, showing that the value of k is reasonably constant.

TABLE 2.

The Effect of Time.

Time of Contact.	Dilution of mixture prior to inoculation.	Average lesions per 0·05 c.c. of dilution.	$k = \frac{1}{t} \log. \frac{V}{v}$
5 minutes	1: 10^4	44·0	·084
10 "	"	17·4	·082
15 "	"	5·0	·090
20 "	1: 10^3	26·0	·082
25 "	"	5·7	·092
30 "	"	2·4	·090

Mixture of equal volumes of virus 1: 10 and 0·1 p.c. formalin.
Control virus dilution averaged 23 lesions per 0·05 c.c. of 1: 10^5 dilution.

(c) *The Effect of Formalin Concentration.*

The rate of the reaction is directly proportional to the concentration of formalin over a wide range of dilution. This relation holds providing that the experiment does not occupy more than one hour, and, a necessary corollary, that extreme dilutions of formalin are not used. Under such conditions, there is a progressive fixation of free formalin by the serum present in the diluent, with consequent diminution in the concentration of formalin available for virus inactivation; as a result the survivors will be in excess of the expectation in the lower concentrations. Titrations of the formalin content of dilutions in the 10 p.c. normal horse serum have shown that this occurs. With the concentrations used in most of the experiments the titratable formalin falls less than 25 p.c. in 30 minutes.

Taken in conjunction with the previous finding, the above relation can be expressed as $Kct = \log. \frac{V}{v}$. Table 3 records a typical experiment.

TABLE 3.

The Influence of Formalin Concentration.

Concentration of formalin in mixture.	Time of contact.	Average lesions per 0·05 c.c. of dilution 1:10².	$K = \frac{1}{ct} \log. \frac{V}{v}$	
0·1 p.c.	3 mins.	91	·157	
„	6 „	43	·106	av. ·119
„	9 „	17	·093	
0·025 p.c.	10 „	162	·138	
„	30 „	34	·092	av. ·104
„	40 „	19	·081	

Formalin dilutions mixed with equal volumes of virus 1: 10.
Control virus dilution averaged 16 lesions per 0·05 c.c. of 10^5 dilution.
0·05 p.c. formalin in the mixture is regarded as unit concentration for calculation of K.

This relation holds over a twenty-fold range of dilution, the maximum which can be readily studied.

(d) *The Influence of Temperature.*

The velocity of the reaction is increased by rise of temperature. Table 4 records a typical experiment. The average value of K at 37° C. is approximately twice that at 23° C.

TABLE 4.

The Influence of Temperature.

Temperature.	Time of Contact.	Dilution of Mixture.	Average lesions per 0·05 c.c. of dilution.	$K = \frac{1}{ct} \log. \frac{V}{v}$	
37°C.	10 mins.	1: 10^2	33	·182	av. ·177
„	20 „	„	0·8	·172	
23°C.	10 „	1: 10^3	17	·101	av. ·092
„	20 „	1: 10^2	48	·083	

0·1 p.c. formalin mixed with equal volumes of virus 1: 10 (unit formalin concentration).
Control virus dilution gave 4·4 lesions per 0·05 c.c. of 1: 10^5 dilution.

DISCUSSION.

The chief point of interest in these experiments is the demonstration of the suitability of the pock-counting technique for dealing with such problems. After one orientating experiment, the influence of the various factors on the reaction was determined in successive experiments which were readily confirmed. The similarity of the findings to those in studies of bacterial disinfection was expected, but could hardly have been demonstrated clearly without a "plating" technique.

In view of the moderately large error of titration, the formula $Kct = \log. \frac{V}{v}$ may be only an approximation. Obviously it will be confined to conditions approximating those of these experiments where the total mass of virus is so small that absorption of formalin to its full capacity will not appreciably diminish the formalin concentration of the mixture. It may be that the relationship is $Ke^{n}t = \log. \frac{V}{v}$ when n is not exactly unity. If so, it can be stated with certainty that n is very close to 1: it does not approach 2 or 0·5. The results are sufficiently accurate to indicate the close similarity of the processes of disinfection by formalin of bacteria and of a typical virus, and no useful purpose would be served by endeavouring to obtain closer approximations to the velocity constants.

SUMMARY.

The kinetics of formalin disinfection of vaccinia have been studied by the chorioallantoic technique.

The relation $Kct = \log \frac{V}{v}$ holds under the conditions of the experiments.

The reaction velocity is approximately doubled by raising the temperature from 23° C. to 37° C.

ACKNOWLEDGMENT.

I have to thank Miss Mavis Freeman, B.Sc., for the formalin titrations.

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THE TEMPERATURE-DEVELOPMENT CURVE OF *LYPEROSIA EXIGUA* DE MEIJERE (DIPTERA, MUSCIDAE) IN RELATION TO THE PROBABLE DISTRIBUTION OF THIS INSECT IN AUSTRALIA

by

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(Submitted for publication 1st May, 1937.)

Lyperosia exigua, the "buffalo-fly", is a small blood-sucking fly about 1/6 inch long. Originally known from the Oriental Region, it has advanced into the Australasian Region and parts of China. It is now widely distributed over Northern Australia, where it is a serious pest of cattle. The flies remain on the host, feeding at intervals. The females lay their eggs in the cattle dung, when it is dropped, and then return to the host; the larvae develop in the dung and pupate either in it or in the surface soil below or near to the dung.

During 1930–32 E. Handschin carried out investigations on the biology of the species in Java and Northern Australia. G. L. Windred (1933) also worked on various aspects of its biology. Handschin (1933a) discusses the probable, ultimate distribution of *L. exigua* in Australia, his conclusions being based on information established during these investigations. They differ considerably from those of Tillyard (1931). Owing to the economic importance of the species in Australia, the writer has re-examined the available evidence with the object of interpreting the data in the light of Handschin's (1933a) and Windred's (1933) observations.

In March, 1933, Dr. A. J. Nicholson, Division of Economic Entomology, Commonwealth Council for Scientific and Industrial Research, asked the writer to analyse the data on which the temperature-development curve referred to by Dr. Handschin (1932, p. 19) had been constructed. He considered that the conclusions regarding the ultimate distribution of *L. exigua* in Australia (Handschin, 1932, p. 20) were based on insufficient evidence, and were incompatible with the known distribution of the species in China. Dr. Nicholson has kindly allowed me to refer to Windred's notes on the Buitenzorg experiments; also notes by T. G. Campbell on the rate of development of *L. exigua*, and some notes on the occurrence of the species in China and Java.

Temperature and Development of L. EXIGUA.

Handschin (1932, p. 20) states: "Results of the work so far done, indicate that temperature controls the spread of the fly, and humidity its local abundance. . . . . Those stations which, for a prolonged period, have monthly mean temperatures of 20° C. or less, may be placed outside the range of permanent occupation of the fly." Rockhampton was stated to be the approximate southern limit of the distribution of the insect in Australia.

The experimental data on which the above conclusions are based are given in a later paper (Handschin, 1933a). The conclusions relating to temperature are based on information from three sources: (1) Experiments on the rate of development of *L. exigua* at different "constant" temperatures, carried out by G. L. Windred at Buitenzorg; (2) Observations by various observers on the time required for *L. exigua* to complete its life cycle; (3) Observations on the geographical distribution of the fly in Java, which indicated that the species does not occur in that country in districts above 1,000 metres altitude, that is, where the mean temperature (monthly) falls below 20° C.

Windred's Buitenzorg experiments were carried out at eight different "constant" temperatures, 0°, 0·75°, 23°, 25°, 35°, 37°, 45°, and 50° C., which were constant within half a degree, except at 23° C. In the latter case, the temperature sometimes rose to 25° C. owing to shortage of ice. Data for complete development of *L. exigua* were obtained at 23°, 25°, 35°, and 37° C. (Table 1). Three experiments were made at each of these temperatures; the periods for complete development given in Table 1 (column 6) represent the averages for the three experiments. These are the figures given by Handschin (1933a, p. 510).

The incubation period for the egg, and the duration of the larval and pupal stage were recorded separately (Table 1). One hundred individuals were used for each stage, in each experiment. The termination of the incubation period for the egg was taken as the mid-time between the hatching of the first and last egg; the completion of the developmental period for the larva was taken as the time when the first larva pupated; and for the pupal stage as the time when the first fly emerged.

Handschin constructed a temperature-development curve for *L. exigua* on the formula $D(T-C) = K$ from the data given in Table 1. He selected the average period for development at 35° C. (10·4 days) and 37° C. (9·6 days) and calculated a developmental zero of 11° C. and a thermal constant of 249·6 day-degrees.[1] The data for development at 23° C. were rejected because "disturbances" were noted during development at this temperature. Those for 25° C. were rejected because fluctuations of two to three degrees about the mean, occurred during the experiments at this temperature.

[1] The formula was based on the Blunk-Bodenheimer formula where D = period of development; T = temperature; C = developmental zero; K = thermal constant.

TABLE 1.

Showing Periods for Development of L. EXIGUA *at Four Different "Constant" Temperatures, obtained by G. L. Windred at Buitenzorg.*

Temp. °C.	Stage.	Experiment No. (hours). 1	2	3	Complete devel. average (days).
23°	Egg	24	22	25	
	Larva	245	260	252	19·5
	Pupa	189	198	193	
25°	Egg	21	22	20	
	Larva	143	142	160	13·5
	Pupa	156	154	158	
35°	Egg	15	16	15	
	Larva	119	120	120	10·4
	Pupa	116	114	118	
37°	Egg	14	14	16	
	Larva	122	100	91	9·6
	Pupa	114	110	115	

The validity of the temperature-development curve given by Handschin depends on the "true" values which can be assigned to the average periods for development at 35° C. (10·4 days) and 37° C. (9·6 days), since these two points only were used in constructing the curve. It is essential that each of these points should represent the "true" mean period for development at the respective temperatures; but the data available from Windred's experiments are inadequate for proving this. For instance, in the egg stage, the mean period for development should be assessed by taking into account the distribution of the hatching periods for all the eggs which hatched from the selected sample of 100 eggs. The periods for development of the larval and pupal stage, in each experiment, are based on one individual in each case. It is unsafe to assign a true value to the mean from these individual observations.

The selected temperatures 35° C. and 37° C. are situated close together; and considerable error in the observed data for development may be expected in this part of the curve. This is particularly the case in these experiments, since the observed average value, for each temperature, is based on three observations only. Moreover, under the conditions of the experiments, 37° C. may be near the upper limit of the linear portion of the temperature-velocity curve. Windred states that, in experiments at this temperature, the flies were poorly pigmented and did not live long; the ptilinal sac often remained distended and the wings filled with fluid.

The available evidence does not justify the rejection of the data for development at 23° C. and 25° C. If we take all the observed values for development at 23°, 25°, 35° and 37° C., the reciprocals lie about a straight line having its origin at — 0·0155. This gives a calculated developmental zero 4·8° C. and a thermal constant 308 day-degrees. The reciprocals of the values for development at 25° C. do not approximate closely to the line. If we reject the data for 25° C., the recip-

rocals of the observed periods for development at the remaining three temperatures approximate closely to a straight line having its origin at — 0·035016; this gives a calculated developmental zero 9·3° C., and a thermal constant 267 day-degrees. If we take the observed values (hours) for development of the egg stage alone, at 23°, 25°, 35° and 37° C., the twelve points lie about a straight line having its origin at — 0·01456, which gives a calculated developmental zero of 6·19° C. and a thermal constant 426 degree-hours.

In constructing the temperature-development curve for the complete life cycle, it is necessary to take into account the fact that the rate of development differs in the various stages of the cycle. In view of the various matters discussed above, the writer considers that further experimental data are required before a reliable temperature-development curve can be constructed for *L. exigua*.

Since Handschin calculated a developmental zero 11° C. for the buffalo-fly, he considers that the lowest temperature at which it will complete its development, must lie between 11° C. and 23° C., the latter value being the lowest temperature for which observed data for development are available. In view of the "disturbances" during development at 23° C., referred to earlier, and having in mind that the fly was not found in localities in Java above 1,000 metres altitude, where temperatures are below 20° C., he concludes that the lowest effective temperature for development is near 20° C. The period for development at this temperature, based on a developmental zero 11° C. and thermal constant 249·6 day-degrees, is 27·7 days. It cannot be inferred from Windred's experiments that 20° C. is the lowest temperature for development of *L. exigua*. This value appears to have been decided upon by Handschin, because of his observations on the range of distribution of the fly in Java, which indicated that it did not occur above 1,000 metres altitude. Dr. Nicholson informs me that the species has been collected on dairy cattle in Java at 1,200 metres altitude; owing to high rainfall and general absence of cattle on the high mountains, "conditions" appear to be unsuitable for the fly. W. S. Patton states that it occurs in parts of China, being common round Peking. The mean temperature for the coldest month at Peking is — 5° C. It would appear therefore that the lowest temperature for development of *L. exigua* extends below 20° C.

The upper limit of temperature suitable for development was fixed at 45° C. The selection of this value appears to be based on a developmental period of 7·1 days recorded by G. F. Hill (Handschin, 1933a, p. 511). The calculated temperature for this rate of development, based on a developmental zero 11° C. and a thermal constant 249·6 day-degrees, is 46° C.

Temperature in Relation to the Geographical Distribution of L. EXIGUA.

On the observations discussed in the previous section, Handschin concludes that the life cycle of *L. exigua* can be completed at temperatures between 20° C. and 45° C., the period of development being one to four weeks. The evidence is not

sufficient to prove that this represents the actual range of temperature for development of this species. Moreover, before the effect of temperature on its development in nature can be assessed from the usual meteorological data, further information is required about the actual temperatures experienced by the immature stages of the insect in dung. When referring to the number of generations which may be expected in the field, based on the temperature-development curve, Handschin (1933a, p. 499) assumes a developmental period of 8 days for Java, giving four generations monthly. This implies a temperature of 42·2° C.; the mean temperature for the warmest month at Buitenzorg is 25° C.

Provided the temperature-development curve satisfies statistical analysis, and is established by taking into account the physiological effects of additional factors in the physical environment, such as moisture, at different stages of the life cycle, it can be safely used to calculate the approximate number of generations which may be expected to develop in an area, in a given time. The degree of approximation will depend upon the degree of correlation between weather data and changes in temperature and moisture in the physical environment of the various stages of the insect. The fermentation heat of cow dung is relatively low, but the temperature of the dung may be high during the day time, due to exposure to the sun. It will vary in this respect according to its moisture content.

T. G. Campbell obtained data at Burnside station, Northern Australia, showing the time required for *L. exigua* to complete its life cycle (Table 2). The insects were reared in the laboratory and the temperatures given represent the average for the various developmental periods.

TABLE 2.

Showing Periods for Development of the Complete Life-Cycle of L. EXIGUA *at Various Temperatures.*

Months.	Dec.	Jan.	Feb.	Mar.	April.	May.	June.	July.
Mean Temperature °C.	28	28·7	27·2	27·3	26·8	25·2	26·2	21·4
Campbell's data (observed)	10	11	13	13	12	15	12	16
When C = 11°C. K = 249·6° (calculated)	15	14	15	15	16	17·5	16	24
When C = 4·8°C. K = 308° (calculated)	13	13	14	14	14	15	14	19

In the absence of further details about Campbell's data, comparisons cannot be made with the calculated data for development given in Table 2. It is of interest, however, to note that the calculated data approximate closer to Campbell's data, when based on a developmental zero 4·8° C. and a thermal constant 308 day-degrees.[2]

[2] R. Melvin and D. E. Beck (J. Econ. Entom. (1931), 24, p. 331) found that *L. irritans* completes its life cycle at 30°C. in 10 days (238·5 hours).

Information is required about the effect of temperature on the adult fly; for instance the minimum temperature at which oviposition can take place. Data are also required about the resistance of the immature stages of the insect to temperatures below the developmental zero. Its occurrence round Peking is evidently due to the ability of the species to over-winter in a resistant stage, in addition to favourable temperatures and adequate rainfall during the summer months; the mean temperature for the warmest month in Peking is about 25° C. Our knowledge about the effect of temperature on *L. exigua* is insufficient to justify Handschin's conclusions (1933a, p. 514) that the species will not become established in Australia in regions where the mean monthly temperature lies below 18° C. to 20° C. for a "long" period of the year.

Moisture in Relation to the Geographical Distribution of L. EXIGUA.

Temperature alone cannot be used as an index to the geographical distribution of the buffalo-fly. It is necessary to consider also moisture "conditions" in the environment of the insect in nature, during the various stages of its development. Handschin (1933a), and Windred (1933) give an account of observations and experiments relating to the effect of dung, having different moisture contents, on the development and mortality rate of the eggs, larvae and pupae. They show that the eggs cannot withstand desiccation. The young larvae prefer dung having a moisture content about 50 p.c.; when saturated, the dung is less favourable for the developing larvae and mortality is heavy. These adverse "conditions" are intensified when flooding occurs. When the dung dries out it becomes unfavourable for the developing larvae, and mortality may be heavy, depending upon the rate at which the dung dries out, and the intensity of dryness. If desiccation is rapid, the larvae may die before attaining the pupal stage; in the case of larvae which develop to the pupal stage, the flies which emerge may be weak individuals. The pupae require less moisture in their environment than the developing larvae. Handschin considers that the pupae do not remain dormant and viable through the dry season in Northern Australia; the insect dies out during the dry months, except in local, marshy situations. With the onset of the rainy season, it multiplies in these situations and spreads outwards. Further experimental data and observations in the field are required, so as to establish precise information about the resistance of the larva and pupa to dryness, and the reaction of the adult fly to atmospheric humidity.

Climate in Relation to the Ultimate Distribution of L. EXIGUA *in Australia.*

It is clear from the work of Handschin and Windred that the moisture and temperature in the physical environment of the insect play a dominating rôle in influencing its abundance and distribution. Biotic competition and the availability of suitable hosts are important additional considerations.

The major elements of climate affecting the moisture and temperature in the environment of the different stages of the insect are rainfall and evaporation, atmospheric temperature and humidity. The former affect the gain or loss of moisture in the dung, the latter affect the activity of the adult fly and the rate of development of the immature stages. The writer (1935–1936) has discussed the importance in Australia of the ratio, *precipitation: atmospheric saturation deficit*, as a guide to the degree of wetness or dryness in the surface soil. With the aid of experimental data and field observations on the drying-out of cattle dung in relation to atmospheric saturation deficit, this formula may afford a useful, general guide to the moisture content of cattle dung in nature.

Tillyard (1931) considered that the 20-inch annual isohyet is the approximate boundary of the probable limits of spread of *L. exigua* in Australia; this view was based on observations on the occurrence of the species in Northern Australia. Later work by Handschin and Windred shows that this interpretation is probably true for Northern Australia, since moisture (rainfall) is the factor limiting the further spread of the insect into the interior of the Continent. It does not apply, however, to the southern portions of Australia, owing to the winter rainfall system which obtains over this region of the Continent; temperature becomes the limiting factor in winter when rainfall is adequate; inadequate moisture (low rainfall) becomes the limiting factor in summer when temperatures are suitable; insufficient moisture (low rainfall) is also the factor limiting its spread inland from the coastal belt. Over the eastern coastal belt and in southern Victoria and Tasmania, where the rainfall may ensure adequate moisture during the greater part of the year, temperature will be the important factor limiting the range of distribution and abundance of the species; in certain situations, excessive wetness may be a contributing factor. The work of Handschin and Windred shows that, in Java, owing to the favourable temperatures, rainfall is a dominating factor limiting abundance of the fly; temperature is the limiting factor at high altitudes. In areas where rainfall is adequate, and temperatures range only a few degrees above the developmental zero of the larva and pupa for several months, heavy mortality may be expected; the vitality of the insect will be low under these adverse conditions, which will be intensified by excessive wetness. This will restrict the abundance of the species and may prevent its permanent establishment in an area.

Tillyard (1931) considered that, within the 20-inch annual rainfall line, "the fly is not likely to be completely killed out, except by heavy frost". In view of the occurrence of the species round Peking, where winter temperatures are below zero, this interpretation appears to be incorrect.

SUMMARY.

The temperature-development curve for *Lyperosia exigua*, given by E. Handschin in 1933, is discussed. It is shown that a reliable curve cannot be constructed from the available data.

The employment of this curve by Handschin, as an aid to the interpretation of the probable geographical distribution of the fly in Australia, is discussed. It is shown that the information on which the curve is based is inadequate for this purpose; further data are required about the influence of temperature on all stages of the insect.

The work of Handschin and of Windred on the effect of moisture on the immature stages of *L. exigua* is briefly referred to. Moisture (rainfall) plays an important part in determining the geographical distribution of the species. In order that the rôle of moisture, in this respect, may be correctly interpreted for Australia, further data are required about the effect of prolonged desiccation on the viability of the immature stages.

The boundaries, illustrating the possible spread of *L. exigua* in Australia, as defined by Tillyard (1931) and Handschin (1933), are based on insufficient evidence; they do not adequately represent the possible ultimate range of the species.

The effect of climate in determining the ultimate distribution of *L. exigua* in Australia may be interpreted in terms of the monthly ratio of *precipitation: evaporation* and temperature. "Low" rainfall limits the spread of the fly into the inland areas of the Continent. Northern Australia is favourable for the insect owing to the summer rainfall system over this region; moisture is adequate during the summer months when temperatures are favourable. The Southern regions of the Continent are unfavourable for the insect owing to the winter rainfall system in these regions. "Low" temperatures in winter, when moisture is adequate, and dryness in summer, when temperatures are suitable, are the limiting factors. In the eastern coastal belt and in the extreme southern portions of the Continent, where moisture (rainfall) is adequate for the greater part of the year, temperature becomes the limiting factor; the southern boundary of the ultimate distribution of the fly in the eastern coastal belt, cannot be determined from the available data.

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THE RESULTS OF THE EXCISION OF THE VENOM GLANDS OF THE AUSTRALIAN TIGER SNAKE *(NOTECHIS SCUTATUS)*

by

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(Submitted for publication 30th March, 1937.)

The experiments recorded here were made to ascertain whether, after removal of the venom glands of tiger snakes, regeneration of gland tissue could occur and what effects ablation had upon the duration of survival of the snakes and on the periodic shedding of their skins.

The venom glands of poisonous snakes secrete a highly specialized saliva, which not only serves for the rapid immobilization of active prey but may also assist in digestion since venom is said to activate the proteolytic enzyme of the pancreatic juice (Delezenne, 1902). According to Phisalix and Bertrand (1894) the glands also contribute to the poisonous qualities of the plasma, which these authors found to be much less toxic in vipers from 52 to 67 days after the glands had been excised. We were, therefore, specially interested to discover the effects of deprivation of the glands upon digestion and upon the toxicity of the plasma.

METHODS.

The venom glands were excised in two series of tiger snakes—the first, 17 in number, in 1930 and the second, 13 in number, in 1934.

The snakes, which were operated on shortly after being captured, were of uncertain age and varied in length between 30 and 48 inches. They were anaesthetized by placing them in a jar containing a piece of cotton wool saturated with ether. Full anaesthesia was obtained in from 15 to 30 minutes. In the first series the snakes' mouths were held open and linear incisions were made, through the mucous membrane of the roof of the mouth, parallel to the margin of the upper jaw on each side. The venom glands were dissected out from behind forwards without opening their capsules, and the ducts were traced as far forward as possible and cut across close to the maxilla. No attempt was made to tie blood vessels since it was found that haemorrhage could be controlled by the external pressure of rubber bands wrapped round the snout but not enclosing the lower jaw. These were left in position for about ten hours. This technique had the additional advantage of obliterating, at least temporarily, the large cavities left by removal of the glands.

In the second series, operation through the roof of the mouth was abandoned because of the almost invariable infection of the wound from the mouth cavity, and the glands were removed through linear incisions on each side from behind the eye through the temporal scales. The glands were dissected out from behind forwards and haemostasis was secured in the same manner as in the first series, but the rubber bands were left *in situ* for from twenty-four to forty-eight hours.

Table 1.

Survival Times After Ablation of Venom Glands.

SNAKES OF SERIES 1.

Series No.	Total Survival in Days.	Testing Periods (Days after operation).	Venom yield by milking.	Gland Tissue found by Section.
HEALTHY SNAKES FROM LAVERTON.				
1	139	—	Nil.	Nil
2	63	—	Nil	Not sectioned.
3	110	—	Nil	L. $2 \times 2 \times < 1$ mm. R. $3 \times 2 \times < 1$ mm.
4	74	Not tested.		L. $4 \times 2{\cdot}5 \times 3{\cdot}5$ mm. R. $3 \times 2 \times 3$ mm.
5	746	244	0·4 mg.	L. $< 3 \times 2 \times 1{\cdot}5$ mm.
		309	0·2 mg.	R. $< 1 \times < 2 \times 2$ mm.
		336	0–2 mg.	
		401	Nil	
		422	Nil	
		533	Nil	
6	83	Not tested.		Not sectioned.
7	>460	244	Nil	A small bud of venom gland on left side. Only duct tissue on right.
		312	0·4 mg.	
		336	0·2 mg.	
8	530	228	Nil	L. $1{\cdot}5 \times 2 \times < 1$ mm.
		278	Nil	R. $4 \times 2{\cdot}5 \times 3{\cdot}5$ mm.
		307	0·4 mg.	
		334	0·8 mg.	
		399	0–7 mg.	
		320	0·7 mg.	
9	467	211	Nil	Small buds of venom tissue continuous with ducts.
		226	Nil	
		276	Nil	
		305	0·1 mg.	
		332	0·2 mg.	
		397	0·3 mg.	
		418	Nil	
10	48	Not tested.		Not sectioned.
11	748	—	Nil	L. Abscess. R. Only necrotic gland tissue $3 \times 2 \times 3$ mm.
SNAKES INFESTED WITH LUNG FLUKE FROM MITTA VALLEY.				
12	199	—	Nil	Not sectioned.
13	628	193	5·9 mg.	L. Nil.
		257	4·5 mg.	R. $6 \times 3 \times 4$ mm.
		360	5·7 mg.	
		381	4·7 mg.	
		493	6·2 mg.	
14	120	—	Nil	Not sectioned.
15	93	—	Nil	Not sectioned.
16	12	Not tested.		Not sectioned.
17	101	—	Nil	Not sectioned.

After operation the snakes were kept separately each in a petrol tin with a wire gauze lid tightly tied down with cord. The room in which they were placed was kept warm. The snakes of the first series were exposed to sunlight on days which were not too hot, but this was not done in the later experiments because of the risk of overheating. The snakes of the first series were allowed water immediately after the operation, but in the second series water was withheld for two or three weeks to diminish the risk of infection. The snakes were fed every month with small mice, and six days after each feeding they were washed and placed in clean sterile tins.

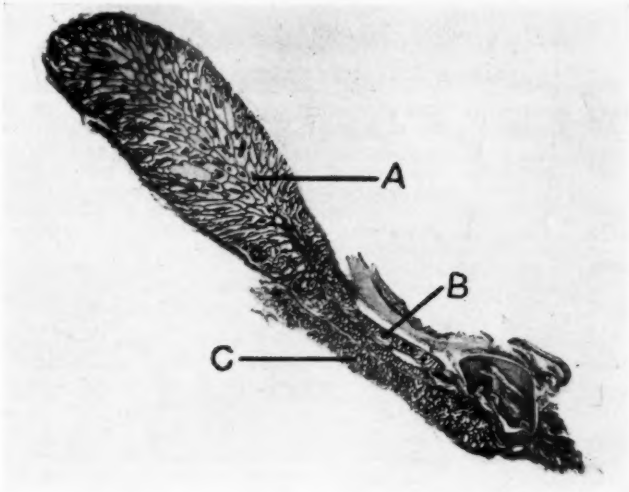

Fig. 1. Photomicrograph of a section of the venom gland and duct of a normal tiger snake. × 5. A, venom gland; B, duct; C, supralabial mucous glands.

In the first series, tests of functional capacity of the remaining gland tissue were made commencing about a month after the operation, at first by allowing the snakes to bite mice (10 to 20 gm. weight), or guinea-pigs (250 gm.) and later by "milking", the venom yields being dried and weighed on a micro-balance. When the snakes died or were killed the amount of venom gland tissue remaining was estimated in serial paraffin sections of the heads. This was done by measuring two axes in the area of gland, the third dimension being provided by multiplying the thickness of the sections by the number in which the gland tissue could be seen. The size of the normal venom gland of a snake of average size would be estimated in this way as 12–14 × 4 × 4 mm.

In the second series the excised glands and those portions of the ducts which had been removed were examined and measured immediately to ascertain the completeness of the operation. Functional tests were made from time to time by allowing the snakes to bite mice. When the snakes died or were killed, the labial, temporal and parietal scales were removed, and blocks of the soft tissues including the muscular beds of the venom glands and the remains of the duct with the surrounding supralabial mucous glands, were dissected out and examined in serial sections.

RESULTS.

The Completeness of Ablation and the Evidence Concerning Regeneration.

In many of the snakes of the first series the operation was not sufficiently radical, though in a few no evidence could be obtained that any functional venom gland remained. In the second series the operation was shown to be complete in almost all the snakes.

TABLE 2.

Survival Times After Ablation of Venom Glands.

SERIES 2. HEALTHY LAVERTON SNAKES.

Series No.	Date of Operation.	Survival in Days.	Examination of Tissues at Death.	Notes.
18	12/4/34	854	L. No gland tissue found R. Tiny bud continuous with duct.	Fed well from 18/6/34. Shed skin, 4/1/35, 8/4/35, 10–16/8/35 and 18/2/36. Killed, 14/8/36.
19	12/4/34	287	No gland tissue macroscopically.	Never fed. Did not shed skin. Died, 24/1/35.
20	17/4/34	23	Not examined.	Death from haemorrhage, 10/5/34.
21	17/4/34	23	Not examined.	Death from haemorrhage, 10/5/34.
22	17/4/34	230	No gland tissue macroscopically.	Only fed once, 3/9/34. Shed skin, 1/10/34. Died, 3/12/34.
23	17/4/34	852	A tiny bud of gland tissue continuous with each duct.	Fed well from 9/10/34. Shed skin, 7/3/35, 23/8/35 and 17/10/35. Killed, 17/8/36.
24	20/4/34	556	No gland tissue macroscopically.	Fed well from 9/10/34. Shed skin, 18/9/34 and 6/2/35. Vomited food, developed swelling of neck, 3/10/35. Found dead, 28/10/35.
25	20/4/34	606	No gland tissue macroscopically.	Fed well from 27/11/34 till 15/4/35. Shed skin, 1/10/34 and 13/7/35. Died, 17/12/35.
26	20/4/34	545	No gland tissue macroscopically.	Fed well from 25/7/34. Shed skin, 4/1/35 and 2/8/35. Died, 17/10/35—swelling of head and neck.
27	20/4/34	66	Not examined.	Did not feed. Canker of mouth. Died, 25/6/34.
28	20/4/34	850	Small bud at end of right duct, fragment in muscle bed on left side.	Fed well from 9/10/35. Shed skin, 4/1/35, 8/4/35. 2/8/35 (incomplete), 30/12/35 and 18/2/36. Killed, 18/8/36.
29	20/4/34	807	A small bud continuous with duct on the left side.	Fed well from 18/6/34. Shed skin, 18/9/34, 15/4/35, 16/9/35 and 24/12/35. Killed, 6/7/36.
30	20/4/34	34	Not examined.	Canker of mouth. Died, 24/5/34.

Some of the data in regard to the first series are summarized in Table 1. Two (4 and 3) of the four snakes of the first series which were bled out to test the toxicity of the plasma on the seventy-fourth and one hundred and tenth days, were able to kill mice or guinea-pigs within times which suggested that the yield was

only a fraction of a mg. of dry venom, and serial sections revealed small buds of venom gland tissue continuous with the ducts on both sides. The other two (1 and 2) bled out on the sixty-third and one hundred and thirty-ninth days, were unable to kill mice or guinea-pigs. In serial sections of the head of the snake, killed 139 days after operation, no trace of venom gland tissue could be found.

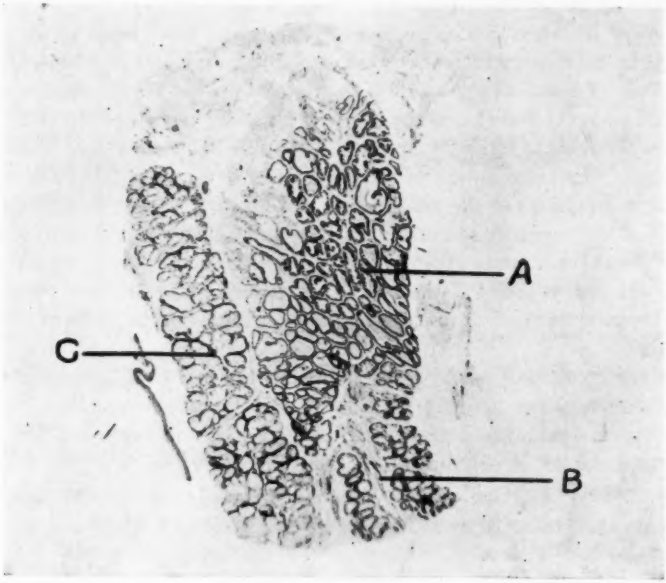

Fig. 2. Photomicrograph of a section from snake 28 showing a bud of venom gland tissue on the right side 850 days after operation. × 60. A, venom gland tissue; B, duct; C, supralabial mucous glands.

The function of the venom glands was tested in all the remaining snakes of the series except No. 16 which died from haemorrhage. Five tested on several occasions (6, 12, 14, 15 and 17) were never able to kill mice. The venom yields therefore could not have exceeded 5γ of dry venom. The heads of these were not sectioned. The remaining seven snakes were able to kill mice in times varying from two to fifteen minutes, in some cases as early as the twenty-fifth day after operation. In one snake (No. 13) yields of about 6 mg. of dried venom were obtained from the right side by milking, and sections of the head revealed a large bud of venom tissue, on the right side, but only duct tissue on the left side. Snakes 5, 7, 8 and 9 yielded from 0·1 to 0·8 mg. of venom on milking, and sections showed small buds of venom gland tissue on one or both sides continuous with the ducts. One snake, No. 11, yielded no venom on milking, and a small necrotic area of venom

tissue was found on the right side. Though the shape of the remaining fragments of gland continuous with the duct suggested repair of the open end of the duct by proliferation of venom gland tissue (compare Figures 1 and 2), in none of these snakes was there any indication during life, either from the death times of bitten animals or from the yields obtained by milking, of continuous increase in the amount of functional venom tissue.

In the second series (Table 2) the operation was shown to be complete in almost every instance. The portion of gland and duct removed at operation, measured in its longest axis, varied from 1·4 to 1·9 cm. In two snakes (24 and 28) a tiny sliver of gland tissue was left in the post-orbital region on one side. The functional tests showed that at no time during their period of survival were any of these snakes able to kill mice; nor was it possible to obtain even a trace of venom by milking. The examination of the soft tissues from the side of the upper jaw, including the remains of the venom duct and its surrounding mucous glands and the whole of the muscular bed of the venom gland, showed at most only a small bud of gland tissue continuous with the duct (Fig. 2). In one snake (28) there was a fragment of venom gland tissue, which corresponded to the tiny piece of gland left in the muscular bed at operation. This did not appear to have increased in size.

The results in this second series decisively negatived the possibility that the venom glands may regenerate after ablation. Furthermore there was no evidence that the supralabial mucous glands, which surround and drain into the duct, undergo compensatory hypertrophy following the operation.

The Effects of Ablation upon the Period of Survival.

Ablation of the venom glands does not appear to affect the duration of life. The average length of life of snakes in which the venom glands were removed was at least as great as that of unoperated snakes kept in captivity and "milked" at monthly intervals. These seldom survived for more than eighteen months, whereas several of the snakes deprived of their venom glands were in excellent condition more than two years after their operation.

The survival times of the snakes of the first series are given in Table 1. There was one death (16) on the twelfth day following several post-operative haemorrhages. Four snakes (1, 2, 3 and 4) were killed by bleeding out to test the toxicity of their plasma. Two (8 and 9) died from over-heating. The survival of the remainder was partly determined by pre-existent disease. The snakes which came from the Mitta River and which were heavily infested with lung fluke *Dolichopera maculpini* (Nicoll), with resultant anaemia of varying degree, had an average survival time which was about half that of the healthy snakes captured at Laverton. An equally important factor was the infection of the operation wounds which was responsible for some early deaths (6 and 10) in this series.

The periods of survival of the snakes of the second series are given in Table 2. There were two early deaths (20 and 21) from haemorrhage and two from cancre (27 and 30). Two snakes (19 and 22) died after 287 and 230 days respectively. The remaining seven snakes, four of which were killed by bleeding out when in excellent condition, survived between 545 and 854 days.

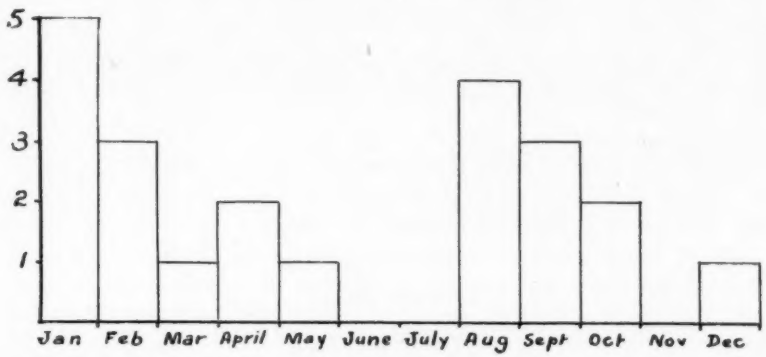

Fig. 3. Graph showing the monthly incidence of shedding of the skin in seven tiger snakes of Series 2.

The Effect of Ablation on the Shedding of the Skin.

Snakes normally shed their skins several times a year. In the second series seven snakes which fed well and survived for long periods, shed their skins three times a year. There was some interference with the normal rhythm of their lives, since they had no period of hibernation at relatively low temperatures, but were kept in a warm room throughout the experiment. In Fig. 3 the number of times that shedding of the skin was observed in these seven snakes is graphed against months of the year. Though the number of moults observed is small it is sufficient to indicate the persistence of the normal rhythm under these abnormal conditions. The graph shows two peaks of activity, one in the spring months (August and September) and one in the late summer months (January and Febr ary). Snakes with intact venom glands do not shed their skins more frequently when kept in captivity.

The normal shedding of the skin in these seven snakes may be taken as an indication of their general health. The snakes were lively in their movements and just as difficult to handle as unoperated snakes. Any difficulty in shedding the skin appeared either to interfere with the health of the snake or to result from ill-health. When the skin was not completely shed, the snakes sometimes refused to eat, or having eaten, vomited their food in an undigested, or partly digested, condition.

The Effects of Ablation upon Digestion.

Removal of the venom glands does not appear to interfere with digestion. In the first series those snakes which survived longest fed well and digested their food, although, as judged by functional tests, in all but two (8 and 13) they had been deprived of more than 99 p.c. of their normal venom supply. All the snakes of the second series which survived over 300 days, though unable to kill their prey, fed readily upon mice which had been killed immediately beforehand by a blow on the head. The excreta did not differ from those of normal snakes and were passed about five days after feeding as in snakes with intact venom glands. Some of our snakes fed regularly throughout the year, taking two or three mice at a meal and completely digesting them. Others ceased to feed, or fed with less avidity, during the normal months of hibernation, but regained their appetite in the spring. A good example of this was snake 18, of which the following condensed protocol is given. The figures in brackets following the dates are the number of mice eaten.

Snake 18. Killed 852 days after operation: 41·25 inches long. 12/4/34, operation.

15/5/34 (1), 3/9/34 (1), 9/10/34 (1), 27/11/34 (2), 4/1/35 to 14/1/35, shed skin. 25/1/35 (3), 7/3/35 (3), 10/4/35 (1), 20/5/35 (0), 30/5/35, shed skin, 13/7/35 (1), 26/7/35 (2), 26/8/35 (3), 2/10/35 (2), 2/10/35–17/10/35, incomplete shedding of skin. 18/11/35 (2), 25/11/35, vomited mice imperfectly digested. 11/1/36 (3), 18/2/36 (2), 24/2/36, shed skin. 21/4/36 (0), 13/7/36 (2), 14/8/36, killed.

It will be noted that on one occasion, associated with incomplete shedding of the skin, the mice which had been eaten were vomited incompletely digested, seven days later.

Delezenne's (1902) finding that the venom may activate the pancreatic juice of some species of snakes has been confirmed for the tiger snake in some unpublished experiments by Holden, who observed, somewhat irregularly, an increase in the proteolytic activity of the pancreatic juice in the presence of venom. This mechanism is obviously useful since poisoned prey is normally swallowed whole and subjected to digestion only when impregnated with venom, but it does not, in the light of our experiments, appear to be essential to ophidian digestion.

The Effect of Ablation on the Toxicity of the Plasma.

In these experiments upon tiger snakes, contrary to the findings of Phisalix and Bertrand on vipers, removal of the venom glands had no effect upon the normal toxicity of the plasma. If plasma is obtained from freshly-caught snakes it contains venom, because snakes almost invariably bite themselves or their fellows during capture or after being caught. If such freshly-caught snakes are isolated for some days in separate cages the venom disappears from the circulation but plasma obtained from them is still toxic. When injected intraperitoneally into guinea-pigs it causes immediate swelling of the abdomen. There is intense congestion of the intestines and of the peritoneal surfaces, and fluid collects in the peritoneal cavity. The animals collapse and die. After doses ranging from 0·5

to 1·5 c.c. in guinea-pigs of 300 grams weight death usually takes place in from two to twenty hours, though after the smaller doses recovery may occur.

The plasma of four snakes (first series) from which the venom glands had been removed, did not differ in toxicity from that obtained from normal tiger snakes after a few days' isolation. These results have already been recorded (1931). In two of these snakes removal of the venom glands had not been complete but the plasma was not more toxic than that of the other two in which all the venom gland tissue had been removed. The plasma of three snakes of the second series, which survived over 800 days after complete removal of the glands, has been examined, and was found to be neither more, nor less, toxic than that of snakes with intact venom glands.

It is evident, therefore, that the toxicity of the plasma in tiger snakes is not due to secretion into the blood stream of any toxic substance from the venom glands, nor to the presence of a precursor of venom which can accumulate when the secretory cells are removed.

DISCUSSION.

The results obtained from the first series of snakes were inconclusive because of the incompleteness of the operation and of complicating sepsis. Though no clear evidence was forthcoming that growth of venom gland tissue was taking place from fragments left at operation, it might well be argued that infection of the operation wounds prevented regeneration which might otherwise have taken place. The results of the examination of serial sections at the end of the experiment suggested that the open end of the duct became closed over by a bud-like proliferation of venom gland tissue, but functional tests during life had not disclosed any progressive improvement in the functional capacity of the glands.

The results of the second series were decisive. Though in four snakes small bud-like masses of venom tissue continuous with the duct were found, there was no evidence that these snakes were able, at any time during their lives, to produce even the 3–5γ of venom necessary to kill small mice. It is certain, therefore, that if removal of the venom glands is complete, regeneration cannot occur from the duct tissue.

These experiments also show that venom is not essential to ophidian digestion. The normal amount injected by these snakes before the removal of the venom glands would vary from 20 to 50 mg. of dry venom, and after operation in the second series the yield was less than 3γ. This trivial amount of venom is unlikely to be adequate to facilitate the processes of digestion, and it seems probable that venom is not essential, since in these snakes digestion of mice was complete in the normal five days and the remains excreted did not obviously differ from the excreta of snakes with intact glands. Possibly the secretion of other glands in the mouth may take the place of venom in this facilitating action, though if this were the

case we should anticipate their compensatory overgrowth during the long period of survival.

Finally, there is no evidence that removal of the venom glands produces any effect upon the normal toxicity of the plasma, from which it may reasonably be inferred that this toxicity is not due to any precursor of venom or to any toxic secretion from the venom glands.

SUMMARY.

The results of ablation of the venom glands in two series of tiger snakes are described. Regeneration of the glands from fragments of duct tissue does not occur, nor is there any obvious hypertrophy of the supralabial mucous glands.

Complete removal of the glands is compatible with survival in good health for over 850 days. The shedding of the skin and the complete digestion of food are still carried out in apparently normal fashion.

The toxicity of the plasma is neither diminished nor enhanced by removal of the venom glands.

Maintaining the snakes under conditions in which hibernation is not possible does not wholly obliterate the normal rhythm of their activities. They take less food and do not shed their skins in winter.

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THE VIRUS OF INFECTIOUS MYXOMATOSIS OF RABBITS ON THE CHORIOALLANTOIC MEMBRANE OF THE DEVELOPING EGG[1]

by

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(Submitted for publication 8th April, 1937.)

INTRODUCTION.

In recent years there has been a recrudescence of interest in the idea, due originally to Aragao (1927), that the virus of rabbit myxomatosis might be a possible means of controlling excessive increase in wild rabbit populations. This possibility is naturally of particular interest to Australia, and on behalf of the Council for Scientific and Industrial Research, Martin (1936) carried out extensive colony experiments in England. He concluded that the virus "satisfied the essential requirements for the control of a population of rabbits in a circumscribed area, and this without danger to domestic animals". In discussing the question of preservation of the virus in an active condition, he suggested that the chorioallantoic membrane technique might be of service, and that the virus should be studied for its ability to grow in this situation.

Martin's work was continued in Australia by Dr. L. B. Bull. In the course of experiments to ascertain the infectivity of the virus for Australian wild rabbits, and to confirm its non-infectivity for other domestic and native wild animals, he also suggested that it should be tested for its power to produce lesions on the chorioallantois of the developing egg. It was thought that failure to grow on the egg membrane would be a useful confirmation of the extreme species specificity of the virus; but to our surprise, the first material tested, subcutaneous tissue from an infected rabbit, gave well-defined foci on the egg membrane similar to those produced by other viruses. The virus showed full infectivity for the egg membrane direct from the rabbit lesion, no egg passage being necessary to produce a satisfactory strain, and, so far as we can detect, no changes in pathogenicity either for rabbit or chick embryo have resulted from continued egg passage.

[1] This work was carried out under a grant for work on virus diseases from the Commonwealth Government Department of Health and the Rockefeller Foundation.

TECHNIQUE.

Twelve-day chick embryos were inoculated with 0·05 c.c. of supernatant fluid, according to the technique described previously from this laboratory (Burnet, 1936, Keogh, 1936). The inoculated eggs were incubated at 36°C., the virus showing more satisfactory lesions at this temperature than at 38·5°C. Passage was made every three days, stock membranes being kept in 50 p.c. glycerol-Ringer solution at 4–5°C. For quantitative experiments, membranes which had been glycerinated for one to four days were used. Dilutions were made from the supernatant fluid in ice cold normal saline containing 10 p.c. of horse serum. "Stock virus" was prepared by grinding one membrane finely with quartz powder, emulsifying in 5 c.c. of nutrient broth, and centrifuging at 3,500 r.p.m. for five to ten minutes.

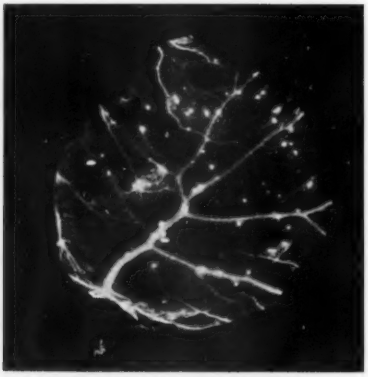

Fig. 1. Myxomatosis lesions on the choriallantois. (Natural size.)

The samples of serum which have been used in the majority of experiments were obtained from a rabbit which recovered after a severe contact infection. Serum M.C.1.A. was obtained during convalescence. The rabbit then received a subcutaneous injection of potent rabbit virus, and serum M.C.1.B. was obtained three weeks later. There was no local or general reaction following the inoculation. Serum-virus mixtures were kept in an ice bath for one hour before inoculation of eggs and rabbits.

THE LESIONS ON THE CHORIOALLANTOIC MEMBRANE.

(1) *Macroscopic appearance.* Membranes removed three days after inoculation with suitably diluted virus showed discrete foci, the appearance of which varied little from egg to egg. They appeared as opaque, raised, whitish areas of proliferation about 0·5–1 mm. in diameter, with clear-cut rather irregular margins. Fully-developed foci were obtained from the original rabbit material, and no change in character has occurred on continued passage. Stock virus suspension prepared as described above regularly gives semi-confluent lesions when used undiluted: diluted 1:1,000 it will usually produce an average of from 3 to 10 foci per membrane.

In infected eggs examined up to six days after inoculation, the embryos were normally developed, and no lesions were observed in them. There may be, however, a certain amount of "leakage" from well-developed membrane lesions, and traces of virus may be found in the liver of the chick. Eggs were inoculated in the usual way with a concentrated emulsion of virus, but were opened from the side opposite the site of inoculation, that is, as far from the area of infected membrane as possible. The embryos were removed intact, washed twice in saline, and the liver removed aseptically for testing. No virus was found in livers 24 hours after inoculation, but in those examined after three and six days' incubation, small amounts were detected.

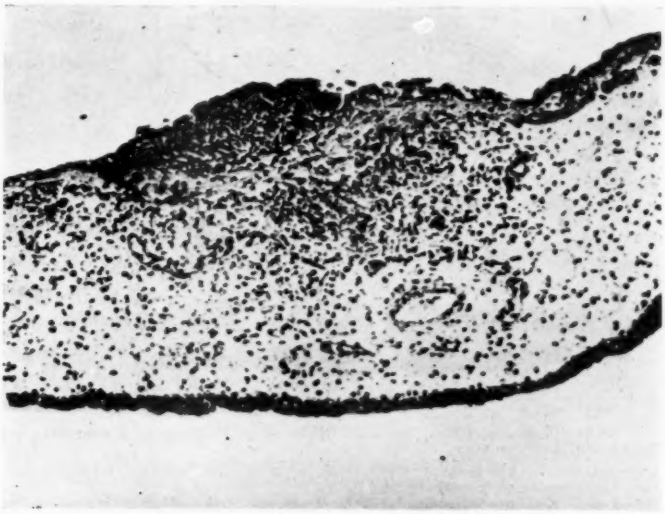

Fig. 2. Three-day lesion showing epithelial proliferation, marked inflammatory response and commencing segregation of necrotic material. (× 120.)

(2) *Histology of Membrane Lesions.* The histology of the lesions follows the general character of other virus lesions in this situation. At 24 hours a few small areas of ectodermal proliferation with, in some cases, a central group of two or three necrotic cells, may be observed. At two days, well-developed proliferative foci are present, the ectodermal cells being rather loosely arranged, and already infiltrated with leucocytes. No inclusion bodies or any appearances resembling the "myxoma cells" of the rabbit have been observed. An active accumulation of cells in the adjacent mesoderm includes eosinophil leucocytes and various histiocytic cells.

Three-day lesions show the full development of the specific focus, and often

early reparative changes. As with most viruses which show only a moderate degree of virulence for the egg, the lesions vary with the degree of resistance of the embryonic tissues to the virus. The more active lesions, which, as is usual, are best seen in younger (ten-day) eggs, and in those incubated at lower temperature, show a loose-textured conical proliferation of ectodermal cells, thickly interspersed with inflammatory cells. Much of the cellular mass is necrotic, particularly on the free

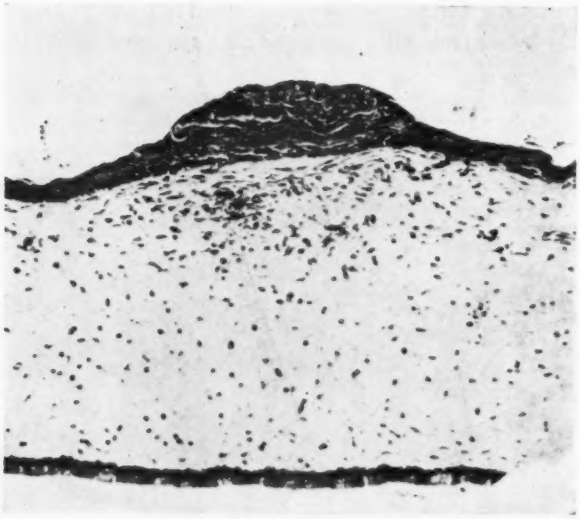

Fig. 3. Five-day lesion showing reconstitution of epithelium and complete segregation of necrotic material. (× 120.)

surface. Toward the mesoderm, the broken-up epithelial cells and leucocytes merge into a dense cellular reaction of the usual type. In lesions where the resistance of the embryo is more manifest, the usual process of epithelial thickening beneath the infected material is taking place, and some foci show a cap of necrotic material well separated from the mesoderm by a zone of healthy epithelium. It is more common, however, at this stage to see the process incomplete, with patches of necrotic leucocyte-filled material throughout the infected ectoderm. In some sections, ineffective reparative growth around such necrotic foci gives a whorl-like appearance: in nearly all there is a more or less distinct cap of completely necrotic debris. If a traumatic ulcer has developed, virus foci developing therein consist of dense spherical accumulations of mesodermal cells packed around a central necrotic zone. No inclusion bodies have been observed in three-day lesions, but the large numbers of acidophil granules from degenerating leucocytes would probably obscure their existence if they were present.

The lesions of five or six days provide a good example of the healing process of the type described for psittacosis lesions by Burnet and Rountree (1935). They differ chiefly in retaining a firmly-textured cap of necrotic material attached to the cornifying epithelium which has segregated it from the other tissues of the

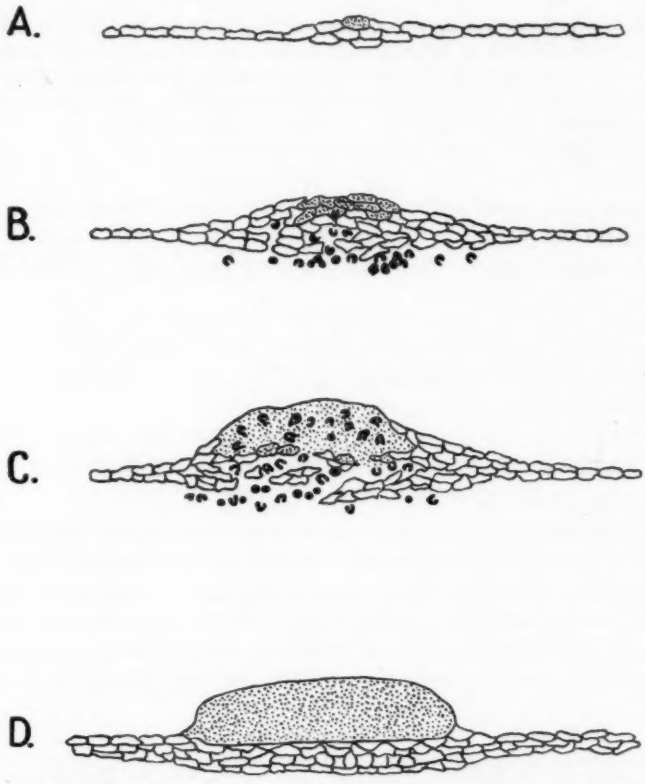

Fig. 4. Diagrams to illustrate the development and retrogression of the ectodermal lesions. A, B, C and D correspond roughly with average one-, two-, three- and five-day lesions respectively. Ectodermal cells are indicated by conventional cell outlines, necrotic material by stippling, leucocytes and other inflammatory mesodermal cells in black.

membrane. Figure 4 shows diagrammatically the types of histological change observed during the development and regression of the lesions. It may be noted here that titration of the virus content from a five- or six-day membrane in the stage of regression showed only from 5–10 p.c. of the amount obtained from comparable three-day membranes.

RETENTION OF PATHOGENICITY FOR THE RABBIT.

Preliminary tests indicated that myxoma would be a suitable virus for quantitative investigation on the chorioallantoic membrane. A "single pock" strain of virus was obtained which has been used exclusively in all subsequent experiments.

Eggs were inoculated with serial dilutions of material from the first generation of egg passage virus. At a dilution of 1: 1,000, one membrane showed two discrete, widely separated foci. One of these was carefully clipped off without removing the membrane from the egg, so as to avoid any chance of "contamination" from the second lesion. The fragment of membrane carrying the isolated pock was emulsified in 0·25 c.c. of broth and further eggs were inoculated. These showed semiconfluent lesions, which provided a satisfactory stock material, and gave rise to the "single pock" strain.

Virus from the first "single pock" generation (S.P. 1) was tested in rabbits by intradermal and intratesticular inoculations. Typical local reactions occurred, followed by generalization, the clinical picture in no way differing from that caused by rabbit passage virus.

Rabbit passage virus from the 26th S.P. generation also gave rise to typical myxomatosis, with death of a rabbit in eleven days. A healthy animal, put into the cage when the first rabbit showed symptoms of generalization (sixth day), showed typical contact infection eight days later.

TITRATION OF THE VIRUS ON THE CHORIOALLANTOIC MEMBRANE AND IN THE RABBIT.

Only well-developed chick embryos were used for quantitative experiments, and virus of the 14th-16th S.P. generations was used throughout. It was found that reasonably accurate titrations could be made by counting the numbers of foci on membranes inoculated with suitably diluted virus. Table 1 shows a typical titration. It will be noted that an occasional membrane shows either a relatively high or low count, but the majority are within 50 p.c. of the mean.

TABLE 1.

Titration of Myxoma Virus on the Chorioallantoic Membrane.

Virus.	Dilution.	Membrane Counts.	Average.
E. 11261 + E. 11262	1: 1,000	3, 8, 2, 3, 4	4·0
	1: 300	17, 15, 18, 8, 30±	17·6
	1: 100	44, 37, 13, 12, 42	29·6

Certain membranes on which numerous secondary foci are present cannot be counted accurately. Where an approximate estimate of primary foci is possible, this is indicated with ± following it.

The relative infectivity of egg passage virus for the egg and for the rabbit was then investigated. The rabbit was found to be slightly more sensitive to the virus than the chorioallantoic membrane. Table 2 shows the results of comparative

titrations in which the rabbits received intradermal inoculations of 0·2 c.c. and the eggs 0·05 c.c. of diluted virus.

TABLE 2.

Comparative Infectivity of the Virus for the Rabbit and the Egg.

Virus.	Dilution.	Rabbits. Inoculum: 0·2 c.c. intradermally.			Membrane Counts. Inoculum: 0·05 c.c.
1. E. 10726 (15 S.P.)	1: 10^6	S	S	S	
	1: 10^5	S	+(7)	S	
	1: 10^4	+(7),	+(7),	+(7)	
	1: 10^3				0, 2, +, 5, 2, 0, 2, 2
	1: 10^2				+, +, 6, 5±
2. E. 11279 (15 S.P.)	1: 10^5	+(7),	+(7),	+(11)	
	1: 10^4	+(7),	+(7),	+(7)	
	1: 10^3				5, 3, 3, 4, 4, 3
	1: 300				17, 8, 13, 14

Rabbit Experiments.

+ ( ). Typical myxoma and number of days before infection generalized.
S. No reaction.

Egg Membrane Titrations.

Certain membranes on which numerous secondary foci are present cannot be counted accurately. Where an approximate estimate of primary foci is possible, this is indicated with ± following it: where no estimate is possible, + is used.

DEMONSTRATION OF THE *IN VITRO* ACTION OF IMMUNE SERUM ON MYXOMA VIRUS.

When mixtures of immune serum and virus are inoculated into the egg, the number of lesions arising is greatly reduced by comparison with those appearing on membranes inoculated with the same virus suspensions as controls. Sera from three rabbits recovering after severe infections of myxomatosis have been tested, and in each case more than 99 p.c. of the virus was inactivated by a 1 : 10 dilution of serum. Table 3 gives typical protocols, and shows the percentage of survivors with different dilutions of serum.

TABLE 3.

The In Vitro *Action of Immune Serum M.C.1.A. on Myxoma Virus.*

Virus.	Serum-virus Mixtures.	Membrane Counts.	Percentage Survivors.
E. 10876	Saline + V/100	39, 37, 42, 49	100·0
	S + V	2, 0, 6, 4	0·07
	S/10 + V	10, 16, 23, 7	0·33
	S/100 + V	50±, 37, 25, 34	9·1
E. 10924	V/1,000	6, 9, 2, 11	100·0
	S/3 + V	3, 8, 5, 10	0·12
	S/10 + V	21, 14, 6, 5	0·33
	S/30 + V	25, 28, 50±, 34	2·2

Certain membranes on which numerous secondary foci are present cannot be counted accurately. Where an approximate estimate of primary foci is possible, this is indicated with ± following it.

Shope (1932) noted that sera from recovered rabbits "neutralized" the virus *in vitro*; rabbits inoculated with his serum-virus mixtures either gave no reaction at all or recovered after a mild infection.

In order to obtain some information as to the relative effects of serum-virus mixtures when inoculated into the egg and the rabbit, a comparative experiment was made, the results of which are given in Table 4.

TABLE 4.

Comparative Titrations of Serum-Virus Mixtures in the Chorioallantoic Membrane and in the Rabbit. Serum M.C.1.B.

	Serum-virus Mixtures.	Rabbits. 0·2 c.c. intradermally.	Average count of four to six membranes. 0·05 c.c.	Percentage Survivors.
A.	Control V/100,000	+(7), +(7), +(11)		
	V/10,000	+(7), +(7), +(7)		
	V/1,000		3·66	100·0
	V/300		13·0	
	S + V/10	+(7), +(7), +(7)		
	S + V/100	S, S, S		
	S/10 + V		8·75	0·48
	S/100 + V		12·0	0·65
B.	Control V/1,000		32·75	100·0
	S + V		29·5	0·18
	S/10 + V		76·4	0·47

Rabbit Experiments.

+ ( ). Typical myxoma and number of days before infection generalized.
S. No reaction.

If we can assume that approximately $1 : 10^5$ represents the limit of infectivity for rabbits, the undiluted serum has reduced this value to 1 : 10, representing the "survival of 0·01 p.c. of infective doses. This is a considerably greater reduction in infectivity than is observed on the chorioallantois. Such differences in apparent neutralizing power of serum, according to the tissue on which mixtures are titrated, are recorded by several authors. Keogh (1936) obtained very similar results in comparing the activity of sera against vaccinia virus on the chorioallantois and by intradermal titiration in rabbits.

DISCUSSION.

These experiments with myxomatosis virus have provided results very similar to those obtained with viruses previously studied in the same way. The maintenance of pathogenicity for the rabbit after repeated passage of a "single pock" strain on the egg can be regarded as showing conclusively that only a single virus is involved in producing the typical rabbit syndrome (cf. Findlay, 1929). Although the susceptibility of the egg membrane to infection by what has been regarded as the most species specific of all viruses is somewhat disconcerting, it

can hardly be used to suggest that the virus has latent potentialities of spread to other species than the rabbit.

Myxoma is a highly specific and infectious virus, difficult and expensive to investigate on an adequate scale when only rabbits are used. The technique described here offers a simple, inexpensive and reasonably accurate method of quantitative study. Such experimental difficulties as the secondary bacterial infection of myxomatous tissues, and accidental spread of virus to stock animals are eliminated.

SUMMARY.

1. The virus of infectious myxomatosis of rabbits produces characteristic lesions on the chorioallantoic membrane of the developing chick.

2. Egg passage virus of the 26th generation produced typical contagious infection in the rabbit.

3. Comparative titrations indicate that the rabbit is slightly more sensitive to the virus than is the chorioallantois.

4. Results of serum "neutralization" experiments in the egg and the rabbit are compared.

ACKNOWLEDGMENTS.

I am deeply indebted to Dr. F. M. Burnet for the advice and assistance which he has given throughout this investigation. I also wish to thank Dr. L. B. Bull for supplying the original material, and making available accommodation at his laboratory for infected rabbits, and Mr. C. G. Dickenson for undertaking the care and clinical observation of these rabbits.

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REGULATED OXYGEN TRANSPORT IN TWO CASES OF CONGENITAL CIRCULATORY DEFECT

by

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Studies of the blood in cases of congenital cardiac defect have been made by Campbell, Hunt and Poulton (1922), Mainzer (1928), Goldschmidt and Light (1925), and Hitzenberger and Tuchfeld (1930). The condition provides an excellent opportunity for studying the regulated transport of oxygen, and in the present instance two cases were examined, one with a simple patent ductus Botalli, and one in which in all probability the only connection between the pulmonary and systemic systems was by means of an inter-ventricular foramen.

Blood Gas Investigations.

Arterial and venous samples were withdrawn simultaneously, the former, by the method of Campbell & Poulton (1922), the latter, after removal of the torniquet in order to avoid artificial alteration of blood gases. It is essential to follow the recommendation of Stadie (1919) both in selection of the size of needle and for sharpening and shaping the point, otherwise it is extremely difficult to pierce the artery and to get a good flow. The skin was locally anaesthetized with Larocain.

The blood was equilibrated at 38°C. for half an hour with known percentage mixtures of oxygen, carbon dioxide and nitrogen in order to determine the dissociation curves, i.e. the characteristics of the blood (Brown & Hill, 1923). The minute volume was estimated by the method of Plesch (1909) and of Eppinger, Papp and Schwarz (1924).

The meionectic curves show that oxygen is more readily given up to the tissues and at a higher tension, a matter of importance in the capillaries. We can with some degree of accuracy reconstruct the conditions existing in the capillaries so far as oxygen is concerned.

The percentage saturation of the blood with oxygen was calculated on the assumption that Hill's equation would give sufficiently accurate results for our present purpose. Hill (1921) and Brown & Hill (1923) as well as Adair (1921) must be consulted for the critical data involved, but substantially the equation of Hill is derived by application of the Law of Mass Action to the equation.

$$Hb_n + nO_2 \rightarrow Hb_n O_{2n}$$

$K [Hb] = [O_2]$ then becomes:

$$K (100 - y) (x) = y.$$

where Hb = 100 − y, y being the p.c. saturation with oxygen and x the oxygen tension in mm. Hg. $[O_2] = \frac{x\alpha}{760}$ where α = the solubility coefficient of oxygen. Since $\frac{\alpha}{760}$ is constant, it may be included in the constant K. n = the average number of haemoglobin aggregates reacting and is here taken as 2·5. Thus we have:

$$(1)\ \frac{1}{K} = \frac{x^{2\cdot5}\,(100 - y)}{y}$$

$$(2)\ \ y = \frac{1 + Kx^{2\cdot5}}{100k \times x^{2\cdot5}}$$

from which, using data obtained by two blood gas equilibrations, the oxygen dissociation curve can be constructed, values for $\frac{1}{K}$ being known. K, which is the oxyhaemoglobin dissociation constant, is determined from the Adair-Henderson graph in which values for CO_2 tension are plotted as abscissae and values for $\frac{1}{K}$ as ordinates. By interpolation, we obtain a value for K corresponding to the closed alveolar CO_2 tension (pulmonary arterial blood) q.v.

The CO_2 combining curves were constructed from the values obtained for CO_2 tension and percentage volume carried respectively, CO_2 concentration being calculated on the basis of complete oxygen saturation, assuming an increase in CO_2 carriage of 0·34 vols. p.c. for each volume per cent. of reduced haemoglobin, cf. Peters, Barr, & Rule (1921).

The hydrogen-ion concentration was determined by the Van Slyke (1921) modification of the method of Hasselbalch (1916), and was calculated for O_2 saturation and 40 mm. CO_2 tension as well as for the actual CO_2 tension existing in the blood in the circulation.

The low oxygen saturations of 64·8 p.c. and 80·0 p.c. found in the arterial blood are evidence for the existence of some short circuiting between the arterial and the venous sides of the vascular system.

TABLE 1.

	Case I.	Case II.	
Total Oxygen Capacity	35·00	35·00	28·6
Haemoglobin—Calc.	25·00	25·00	21·3
R.B.C.	7×10^6	$7{\cdot}8 \times 10^6$	$6{\cdot}1 \times 10^6$
Viscosity	17·0	17·0	15·0
Arterial Oxygen Vols. p.c.	22·61	29·00	25·9
„ CO_2 „ „	28·80	30·05	30·7
Venous Oxygen „ „	11·80	17·82	19·3
„ CO_2 „ „	38·28	41·84	41·0
Arterial Saturation p.c.	64·80	80·0	90·6
Venous „ „	33·71	50·9	67·5
O_2 Tension Pulm. Art.	60·5	31·0	—
CO_2 „ „ „	36·00	38·7	39·68
O_2 „ „ Vein	Alveolar Air	102 mm. O_2	
CO_2 „ „ „	28·17	27·91	36·17
Circulation Time (secs.)	100·0	104	—

Oxygen Equilibrations.

	O_2 p.c.	O_2 Partial Press.	Vols. p.c. Stan.	CO_2 Tension.
Case I	97·5 p.c.	74·2 mm.	90·4	35·7
	81·8 „	61·6 „	83·1	36·3
	64·0 „	48·4 „	72·0	35·0
	95·8 „	71·7 „	85·6	40·0
Case II	75·5 „	56·3 „	75·2	40·0
	60·0 „	44·4 „	62·4	38·5

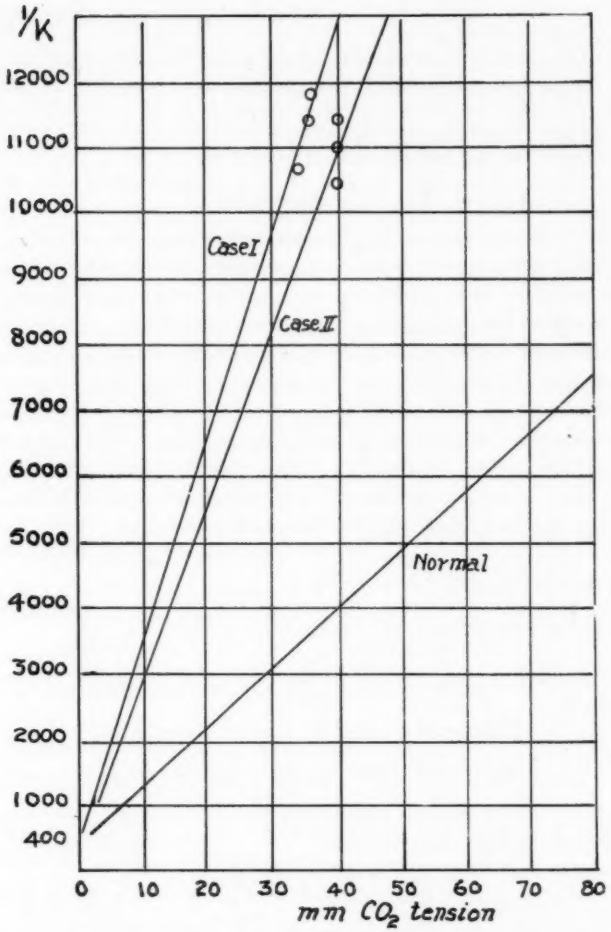

Fig. 1. Adair-Henderson Graph to determine 1/K.

The arterio-venous differences were 22·61 — 11·80 = 10·81 vols. p.c. (30 p.c. saturation) and 28·00 — 17·82 = 10·18 (29·2 p.c.) respectively, and the utilization of oxygen was 47·7 vols. p.c. and 35·6 vols. p.c. These values are strictly true only for the blood in the vessels punctured, but are very approximately true for the total circulation and indicate, of course, an extremely slow passage of blood through the tissue capillaries, giving time for extensive reduction in both cases.

The arterial carbon dioxide content is, compared with normal, very low. Van Slyke (1922) quotes as normal 43–55 vols. p.c. at a tension of 40 mm. Hg. The alkalosis existing in these

cases is due to excessive "washing out" of CO_2 in the lungs and the mechanism of this can be deduced from the following facts:

The CO_2 absorption curves lie below the lowest normal limits given by Van Slyke (1921), and in Case I the curve is almost a straight line coincident with the pH 7·10 line. Since at 40 mm. tension of CO_2 a normal arterial blood would contain 43–55 p.c. by volume of CO_2 (vide graph), and since in actual fact the blood of Case I contains only 30·9 vols. p.c., the blood must have an abnormally low pH—which actually turns out to be 7·1 instead of 7·3.

Deductions Concerning the Nature of the Defects.

Case I. From the table it will be seen that the oxygen saturation in the pulmonary arteries is 80·5 p.c. In the radial artery it is only 64·8 p.c., whilst in the pulmonary veins the blood is 92·0 p.c. saturated. This must mean that venous blood is mixing with the oxygenated blood returning from the lungs. To explain why the pulmonary arterial blood has a higher oxygen content than aortic blood, we must consider the possibility of the aorta rising partially or wholly from the right ventricle, and the pulmonary artery from the left ventricle. Together with a septum defect, this will provide an explanation. As a result a mixture of venous and arterial blood is forced into the aorta, whilst a richer oxygenated blood is forced into the pulmonary circulation (Hitzenberger and Tuchfeld, 1930).

Applying the Fick Principle we obtain the following values for the minute volume.

In the pulmonary circulation we have:

Amount of oxygen in 100 c.c. of blood in left auricle $\frac{92{\cdot}0 \times 35{\cdot}0}{100} = 32{\cdot}2$ c.c.

Amount of oxygen in 100 c.c. blood in pulmonary artery $\frac{80{\cdot}5 \times 35{\cdot}0}{100} = 28{\cdot}17$ c.c.

Difference = 40·3 c.c. O_2 per litre of blood.

$$\text{Minute volume} = \frac{250}{40} = 6{\cdot}3 \text{ litres.}$$

In the systemic circulation we have:

Oxygen in 100 c.c. aortic blood	22·61 c.c.
Oxygen in 100 c.c. right auricular blood	11·80 c.c.
Difference	108·1 c.c. O_2 per litre blood

$$\text{Minute volume} = \frac{250}{108} = 2{\cdot}3 \text{ litres.}$$

It is evident that the blood in the aorta cannot come entirely from the right auricle, since mixed blood is present. To the blood coming from the right auricle is added a definite quantum via the septum defect from the left ventricle, i.e. from the left auricle. The same reasoning applies to the blood entering the pulmonary artery from the left ventricle. We have, therefore, proceeding from the left to right:

$$O_2 \text{ Aorta} = (1 - x)\ O_2 \text{ Vein} + xO_2 \text{ via septum}$$
$$\therefore\ x = 0{\cdot}533$$

i.e. approximately 53 p.c. of the aortic blood comes from the left ventricle.

From right to left we have:

O_2 pulmonary art. $=(1 - x)$ O_2 left auricle $+ xO_2$ right auricle,

i.e. 19 p.c. of the pulmonary arterial blood comes from the right heart.

According to the above assumptions an equal amount of blood passes both ways via the defect. The total quantity ejected from the left ventricle per minute $= 6{\cdot}3 + 1{\cdot}20$ litres $= 7{\cdot}5$ litres. That of the right ventricle per minute $= 2{\cdot}3 + 1{\cdot}6$ litres $= 4{\cdot}00$ litres. The total ejected therefore $= 11{\cdot}5$ litres, of which $6{\cdot}3 + 2{\cdot}3 = 8{\cdot}6$ litres is the circulating quantity, whilst the difference, i.e. 2·90 litres, is thrown from right to left ventricle, and vice versa, by wasteful effort. cf. Hitzenberger.

In *Case II*, we have a typical instance of patent ductus Botalli in which the lungs are to a certain extent short-circuited by the combined effect of the patent ductus, and the predisposing stenosis of the aorta. The degree of oxygen saturation of the blood leaving the lungs is 95 p.c., whilst in the radial artery the blood is 80 p.c. saturated, indicating again a mixing of arterial and venous blood, but to a smaller degree than in the former case.

In the pulmonary circulation we have:

Amount of oxygen in 100 c.c. blood in left auricle $\dfrac{95{\cdot}0 \times 35{\cdot}0}{100} = 33{\cdot}2$ c.c.

Amount of oxygen in 100 c.c. blood in pulmonary artery $\dfrac{48{\cdot}0 \times 35{\cdot}0}{100} = 16{\cdot}8$ c.c.

Difference $= 164$ c.c. O_2 per litre of blood.

$$\text{Minute volume} = \frac{220}{164} = 1{\cdot}34 \text{ litres.}$$

In the systemic circulation we have:

Amount of oxygen in 100 c.c. blood in aorta	28·0 c.c.
Amount of oxygen in 100 c.c. blood in right auricle	17·8 c.c.
Difference	102 c.c. O_2 per litre of blood

$$\text{Minute volume} = \frac{220}{102} = 2{\cdot}1 \text{ litres.}$$

Because of the short circuit, some of the blood in the aorta comes from the pulmonary artery, the fraction x being determined as follows:

Arterial saturation $= 80{\cdot}0$ p.c.

$80{\cdot}0$ p.c. $=(1 - x)94 + 50{\cdot}9x$

$\therefore x = 0{\cdot}325,$

i.e. approximately 32·5 p.c. of the blood in the aorta comes from the ductus arteriosus, or 700 c.c. per minute. This leaves 640 c.c. per minute flowing through the pulmonary area to become fully aerated by carrying 220 c.c. of oxygen per minute.

According to Van Slyke and Lundsgaard (1921) the limiting capillary per-

centage of reduced haemoglobin necessary to produce cyanosis, is between 6 and 7 p.c., corresponding to about 5 gm. of reduced haemoglobin per cent.; above this level distinct colouration of the skin is evident. The quantity of reduced haemoglobin can be calculated as follows:

$$\text{Capillary reduced haemoglobin} = \frac{\text{Arterial reduced Hb} + \text{Venous reduced Hb}}{2.}$$

	Case I	*Case II*
Arterial reduced Hb	$= 35{\cdot}0 - 22{\cdot}61$	$= 35{\cdot}0 - 28{\cdot}0$
	$= 12{\cdot}39$	$= 7{\cdot}0$
Venous reduced Hb	$= 35{\cdot}0 - 11{\cdot}80$	$= 35{\cdot}0 - 17{\cdot}8$
	$= 23{\cdot}2$	$= 17{\cdot}2$ vols. p.c. O_2
	(expressed in vols. p.c. O_2)	
$\therefore$ capillary concentration of reduced Hb	$= \frac{23{\cdot}2 + 12{\cdot}4}{2} = 17{\cdot}8$	$\therefore$ capillary concentration of reduced Hb $= \frac{17{\cdot}2 + 7{\cdot}0}{2} = 12{\cdot}1$

The following results from a normal subject are given for comparison (cf. Hitzenberger and Tuchfeld):

Arterial reduced Hb $= 21{\cdot}05 - 20{\cdot}40 = 0{\cdot}65$
Venous reduced Hb $= 21{\cdot}05 - 16{\cdot}00 = 5{\cdot}05$

$$\text{Capillary concentration of reduced haemoglobin} = \frac{5{\cdot}70}{2} = 2{\cdot}85$$

It will be readily appreciated from these figures that there is excess of reduced haemoglobin in the arteries of both cases before the tissues have withdrawn any oxygen, and notwithstanding the quantity of oxyhaemoglobin resulting from the polycythaemia, the cyanosis is evident.

By comparing the CO_2 absorption curves with those obtained by other workers for bicarbonate solutions, true plasma, blood, and washed corpuscles, Hasselbalch (1916), Van Slyke (1921), Liljestrand (1928), Joffe and Poulton (1920), we can see at a glance what the factors are which cause the abnormal absorption relations between carbon dioxide tension and percentage absorbed. As we pass from a solution of pure bicarbonate to a suspension of corpuscles we find the absorption curves flattening more and more—i.e. in proportion to the degree of buffering, cf. A. V. Hill (1921).

The higher the carbon-dioxide absorption curve, the larger the quantity of bicarbonate present. The low absorption for corpuscles is due mainly to their content of haemoglobin.

Since the buffer value of whole blood is chiefly dependent on its haemoglobin content, Van Slyke (1921), the increased haemoglobin content in the case in question must flatten the curve and move it towards that of a corpuscular suspension. This is the more understandable since the proof by Ferguson and Roughton

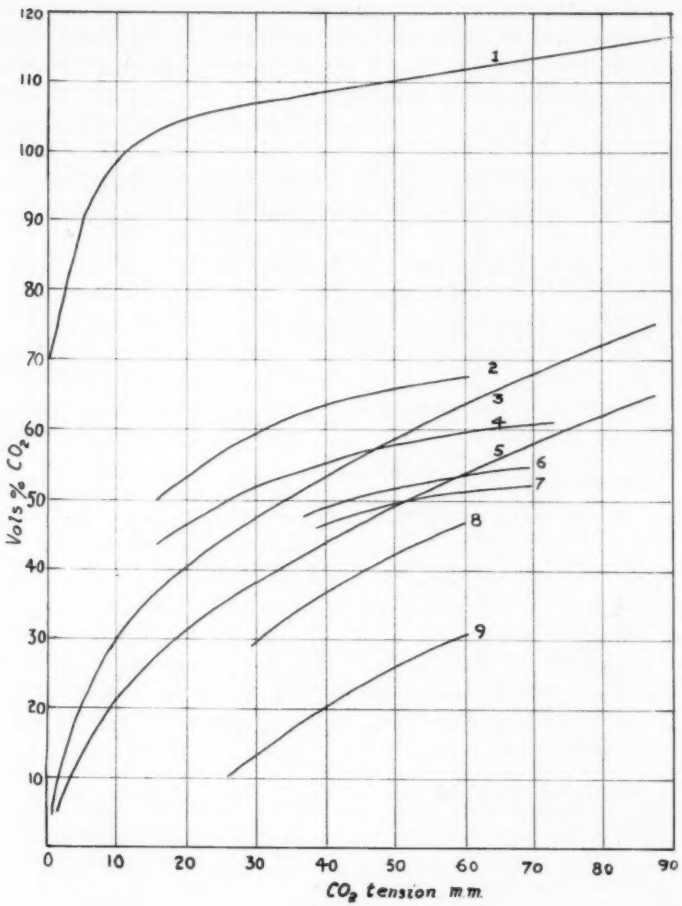

Fig. 2.

1. $NaHCO_3$ Solution.
2. Calculated for Case I (see text).
3. Normal Blood.
4. Normal Serum.
5. Normal Plasma.
6. Plasma, Case II.
7. Plasma, Case I.
8. Blood, Case II.
9. Blood, Case I.

(1934) of the formation of carbamino compounds by haemoglobin and carbon dioxide, to an extent such as to account for a *large proportion of the physiological interchange.*

As the curve becomes more of a straight line, it will cross fewer pH isopleths,

and since in Case I it almost corresponds with the line for pH 7·1, it means that with increasing CO_2 content there is no change in pH—i.e. *that the blood is almost perfectly buffered for acid.* In Case II, the curve sweeps over a pH range of only 0·05 and the same observations apply to it.

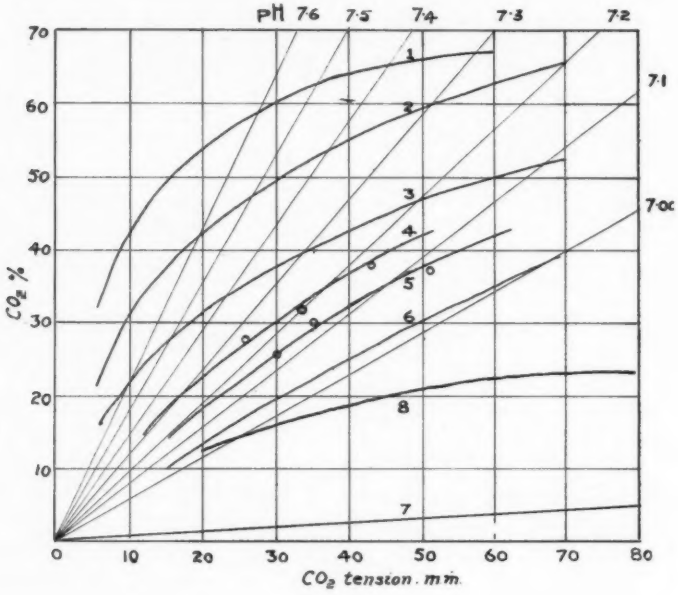

Fig. 3.

1. Calculated value for Case I.
2 and 3. Normal Limits.
4. Case II.
5. Case I.
6. Hitzenberger's Case.
7. Carbon dioxide solubility in water.
8. Carbon dioxide in corpuscles (calculated) whole blood (Joffe & Poulton, q.v.).

Hydrogen-ion Concentration.

Using the equation of Hasselbalch, we have:

$$\text{For Case I, } pH = 6{\cdot}15 + \log \frac{\text{Total } CO_2 - 0{\cdot}1316xp}{0{\cdot}1316xp} = 7{\cdot}10$$

where p = 40 mm. for reduced pH.

Calculating similarly,

For Case II, pH = 7·2.

In the present cases the reduced pH of 7·1 and 7·2 indicates extensive acid buffer-

ing, and should be compared with the values cited by Van Slyke, for uncompensated alkali deficit. It has already been pointed out that the carbon dioxide absorption curves are very much flattened, which indicates that with increasing carbon dioxide content, the buffering permits only slight changes in pH, so that there is no evidence of an uncompensated deficit in these cases, and it is obvious that the adjustment is made by increasing the haemoglobin content of the blood, cf. Straub (1924), Meier (1924), Hitzenberger and Tuchfeld (1930).

In contrast to a normal blood, in which there is almost complete oxygenation, and where the carbon dioxide pressure is 40 mm., there is here a low carbon dioxide tension and a high grade reduction of haemoglobin, a combination of circumstances which would slightly raise the actual pH.

There is thus a greater difference between the regulated and reduced pH values than is normally found to be the case, cf. Hasselbalch (1916). Compared with a subject who, under Van Slyke's classification would border on coma (see Fig. 3), it will be seen from the following calculated pH values that the condition of these subjects is quite different.

	Case I	*Case II*
Blood leaving lungs	pH 7·14	7·35
Arterial blood (Radialis)	7·10	7·21
Venous blood (Cephalic)	7·11	7·33

In order to determine the relative importance of the parts played by corpuscles and plasma in this phenomenon, the carbon dioxide combining curves for the plasma were constructed. From the diagram it will be seen that they lie lower than that for normal serum, although higher than those for the patients' whole blood. According to Joffe and Poulton (1920) the difference in pH between serum and whole blood at 40 mm. is $7{\cdot}38 - 7{\cdot}34 = 0{\cdot}04$, corresponding to a difference of 10 vols p.c. of CO_2.

It will be seen from the absorption curves for blood and plasma in these two cases, that there is a carbon dioxide difference of 17 vols. p.c. and 13 vols. p.c. respectively, measured at 40 mm. Hg tension. This difference is due to the increased haemoglobin content.

It was shown by Bohr (1904) that the quantity of oxygen absorbed by haemoglobin at constant pressure depended upon the amount of carbon dioxide present. Increase in carbon dioxide pressure lowers the oxygen carrying capacity at any given oxygen tension. In the cases now under consideration, owing to excessive buffering against hydrogen ions—which from whatever source are the real cause of the phenomenon, Barcroft and Orbeli (1910), Rona and Ylppö (1916), and Barcroft and Murray (1923)—we might expect little change in oxygen carrying capacity due to alteration in carbon dioxide pressure. This is actually the case, and although we have dissociation curves displaced as a whole towards the right, we find that owing to excessive buffering there are only slight changes caused by

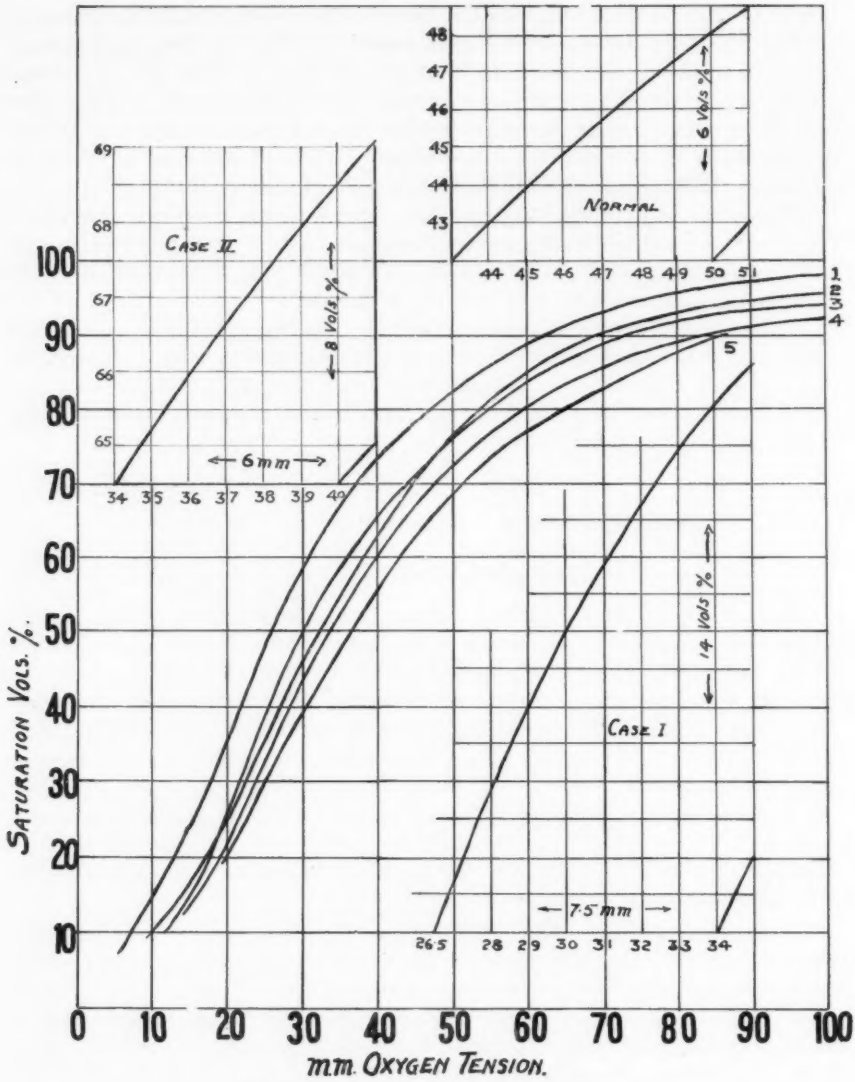

Fig. 4. Haemoglobin Dissociation Curves.

1. Bock's Blood pH 7·4.
2. Bock's Blood pH 7·2.
3. Case I.
4. Case II.
5. Blood pH 7·1.

The inset curves show the amounts of oxygen liberated for a given change in tension for normal blood, and for Case I and Case II at their respective capillary oxygen tensions, as compared with normal blood.

variations in the carbon dioxide tension. This was demonstrated by Hitzenberger and Tuchfeld in their own case.

Haemoglobin Dissociation Curves and Oxygen Transport.

	Normal	*Case I*	*Case II*
Average Concentration of Reduced Haemoglobin in Capillaries	3·45 vols. p.c.	17·5 vols. p.c.	12·1 vols. p.c.
Total Oxygen Capacity	21·19 „	35·0 „	35·0 „
Difference	17·74 „	17·5 „	22·9 „
Percentage Saturation	83·7 p.c.	50 p.c.	65 p.c.
Oxygen tension in Capillaries	50 mm.	34 mm.	40 mm.

It will be seen from the dissociation curves for haemoglobin that the following are the relationships existing between oxygen tension and percentage saturation of the blood.

At 55 mm. O_2 Tension.

	Tension Difference (mm. Hg.).	Saturation Difference Vols. p.c.
*Normal	5	3·5
*Bock's Blood pH 7·2	5·0	5·0
Case I	5·0	5·0
Case II	5·0	5·5

At 40 mm. O_2 Tension.

Case I	5	6·6
*Bock's Blood pH 7·2	5·0	6·6

At 34 mm. O_2 Tension.

Case II	5	10

* Taken from Peters and Van Slyke, Vol. 2, 1931.

It is obvious that at the capillary oxygen tensions existing, the patients I and II secure almost two and three times as much oxygen respectively, for the same fall of oxygen tension, as in the normal case. The effect of hydrogen-ion concentration in normally constituted blood shows the effect of meionexy to be approximately the same in this respect.

These figures give some basis for discussing the adjustment of the oxygen transport to the abnormal circulatory conditions, *vide* the dissociation curves (Fig. 4). At the low oxygen tensions existing in the capillaries we find that since we are dealing with equilibria depicted by the steep portion of the curves, there is more oxygen liberated for the same drop of pressure than at normal capillary pressures. This, to some extent, may be looked upon as neutralizing the adverse effect of diminished action of carbon dioxide upon oxygen carriage in these regions (*vide* Table).

TABLE 2.

Gas Mixtures.

Per cent. O_2.	Per cent. CO_2.	Partial Press. O_2.	Partial Press. CO_2.	Blood Vols. p.c. O_2.
6·6	6·4	49·2	41·2	26·0
6·5	4·05	51·2	30·4	28·2

Difference due to meionexy = 2·2 Vols. p.c.
Cf. Hitzenberger & Tuchfeld. Normal = 3·6 p.c.

The low grade Bohr effect thus indicated is to be expected in the presence of such excessive buffering for hydrogen ions. This condition, however, must be of considerable advantage to tissues which are being supplied with oxygen at a low pressure gradient in many instances. The excessive buffering effect of the haemoglobin is obviously not mainly connected with the Hamburger phenomenon, and may be traced to the formation of carbamino compounds with some of the available reduced haemoglobin, as recently demonstrated by Ferguson and Roughton, loc. cit. *In other words, by meionexy and by low tissue oxygen tension together, a greater total yield of oxygen is obtained—a result contributed to by the slower circulation.*

The greater yield of oxygen, however, is not itself sufficient to restore conditions in the tissues to normal, since the driving force behind the oxygen is the pressure gradient which is, in this case, much reduced. In the normal case cited for comparison, there was an average capillary oxygen tension of 50 mm., whilst in the capillaries of the patients under examination, there are only 34 mm. and 40 mm. pressure, respectively. Using the diagrammatic scheme of Krogh we can show to what extent the tissues suffer from hypoxaemia (Fig. 5). The distance R to which an oxygen molecule will travel from the central capillary c. into the surrounding cylinders of tissue, depends upon the pressure gradient of the oxygen. If the circles overlap all cells secure oxygen, if they merely touch there is a triangular area in which lie cells which suffer from anoxia. From what has been stated earlier, it is clear that in these cases the arterial limb of capillary loop provides an oxygen tension comparable with that existing in the *venous* limb of a capillary of a normal individual. The following formula worked out by Erlang, for Krogh (1922), gives the relation between the data available:

$$T_o - T_R = \frac{10^4 p}{d}\left(1{\cdot}15R^2 \log. \frac{R}{r} - \frac{R^2 - r^2}{4}\right)$$

where T_o and T_R are the oxygen tensions at the capillary and at the distance R respectively.

R = Radius of cylinder of tissue supplied by oxygen from the central capillary of radius r.

d = Diffusion constant for O_2.

p = No. of c.c. of oxygen used per minute by 1 cubic millimeter of tissue.

Krogh was able to make use of this equation to calculate the approximate relations

in muscle tissue at rest, under massage, at work, etc. We are unable to make the same assumptions from the microscopic anatomy, but we can indicate that as the oxygen consumption rises, the formula shows that the value for R must decrease, whilst that for r must increase. At the same time $T_O - T_R$ must fall steeply. The effect of this upon tissues will vary with their resting oxygen tension, but the

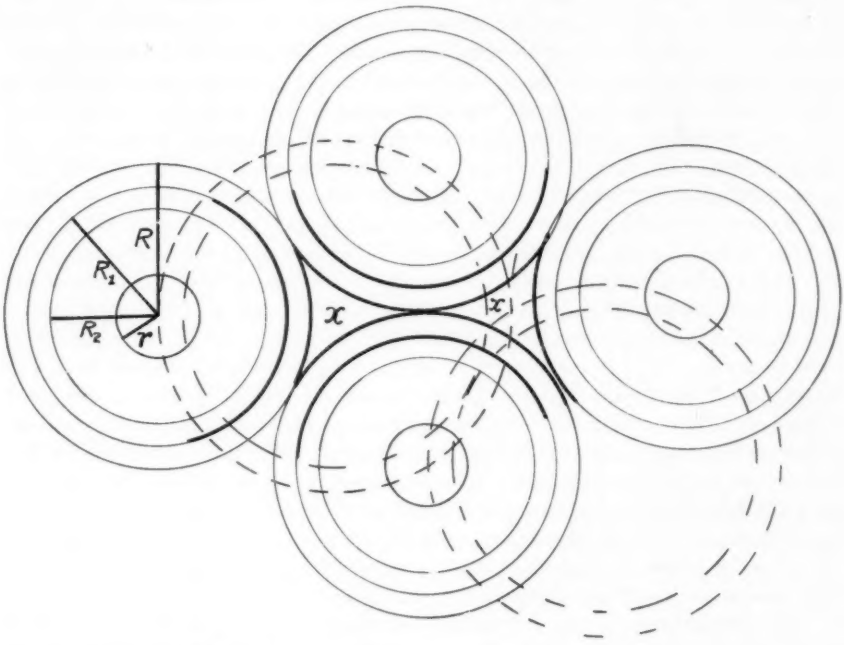

Fig. 5. Schema to illustrate oxygen supply to tissues.

r = radius of capillary.
R = radius of tissue cylinder served by normal arterial oxygen pressure.
R_1 = radius of tissue cylinder served by arterial oxygen pressure, Case I.
R_2 = radius of tissue cylinder served by venous oxygen pressure, Case I.
x = Anoxic area of resting muscle served by new capillaries whose oxygen supply is indicated by broken circles, the larger of which represent conditions in activity.

capillary response, as shown by examination of the nail bed, etc., in these cases, shows that the defect is counteracted by the opening up of more capillary loops. Instead of increased tissue activity being the cause of increased capillarization, as in Krogh's studies, we have here a diminished minute volume acting in the same sense.

In resting muscle, for example, the capillary supply of which was studied quantitatively by Krogh, whilst the resting oxygen consumption was also measured by Verzár (1912) and by Gaarder (1918), the suggestion is that there are portions of tissue lying between those cylinders which are supplied by their central capillaries, and which therefore obtain no oxygen. The continual opening up of new loops, and disappearance by constriction of old loops, keep the tissues supplied throughout, over any period of time. In our cases this mechanism is not sufficient, as the diagram, using the method of Krogh, attempts to show; therefore, even at rest, more loops on the average, must be open. How is it possible for the tissues to secure oxygen in such adverse circumstances? There are several compensating mechanisms: (1) The shift to the left of the whole of the curves favours reduction of oxyhaemoglobin by bringing the steep portion of the dissociation curve into the range of oxygen tension of "normal" capillary interchange. It is obvious that in the lungs the shift introduces an adverse factor, since higher oxygen tension is necessary to secure a corresponding degree of saturation. The alveolar oxygen tension of 102·23 mm. however is sufficient to bring into operation the conditions depicted by the upper flat section of the curve. Here, it will be seen, the adverse effect of the meionectic shift of the hamoglobin dissociation curve plays an insignificant rôle. It is interesting to compare with these cases the conditions found to exist in anoxia due to low atmospheric oxygen tension such as obtain at high altitudes. Here the subject adjusts his blood transport system by moving the dissociation curve of haemoglobin *to the right*. This has the reverse effect of increasing the ease of oxygen uptake in the lungs and of diminishing the ease of dissociation in the tissues. The former effect however overshadows in physiological importance the latter result (Barcroft, 1928, 1929). In our patients, owing to high alveolar oxygen tension, there is no difficulty in securing oxygenation of haemoglobin in the lungs; the important thing is to accelerate the dissociation of oxyhaemoglobin in the tissue capillaries.

(2) Owing to the severe degree of hypoxaemia in these cases, the reduction of the oxyhaemoglobin takes place under conditions summarized graphically by the steep portion of the dissociation curve. The arterio-venous differences in these cases were 10·8 and 10·2 vols. p.c. The normal value lies between 5 and 6 vols. p.c. To achieve this it is only necessary to point out that *twice the normal pressure drop would be required*—other conditions remaining of course the same. It is also evident that there is considerable advantage accruing from increased total oxygen capacity in this connection.

(3) There was microscopic evidence from examination of the fundus and nail bed capillaries that there were more capillary loops opened up. This also ensures more rapid passage of oxygen outwards from the capillary bed. Krogh (1922) demonstrated this effect of anoxia on the capillaries of tissues, and proved that ordinary active hyperaemia increases the available capillary surface by ten times the resting value.

(4) The increased oxygen utilization, i.e. 47·7 p.c. and 36·5 p.c. respectively,

compared with the normal of 22 p.c. indicates a slower circulation, the cause of which is obviously the high viscosity due to the increased number of corpuscles (Hess, 1920). The polycythaemia therefore not only ensures a greater *total* oxygen supply, but it provides a mechanism for better utilization of the oxygen which is carried to the tissues. This compensatory effect, however, brings with it some disadvantages. The increasing load on the heart due to rising viscosity ultimately puts a limit to the cardiac capacity, whilst increasing circulation time slows the *rate* of oxygen supply to the periphery. The case of patent ductus Botalli cited provides ample clinical evidence of this, for cardiac weakness, shown by accelerated and weak pulse, breathlessness, etc., culminates in an "attack" with haemorrhage from the lungs, and menorrhagia. After some weeks of rest in bed, with or without oxygen, the subject is restored sufficiently to carry on again a limited "normal" existence, but with a lower erythrocyte count. The transport regulation in the blood is thus seen to be defeated by the mechanical failure of the cardiac mechanism.

(5) On the other hand, the slow circulation itself will mean *less rapid removal of* CO_2 from the tissues, and this defect is met by increased haemoglobin content of the blood. In fact, but for the buffering so provided, the patients' condition would be quite as troublesome from this cause, as from failure to increase the red corpuscle content.

The clinical history of sleeplessness and finally of paroxysmal dyspnoea in Case II is, moreover, the beginning of the condition of permanent over-ventilation which leads to the low alveolar CO_2 shown to be present, and which is probably due to anoxia of the carotid sinus end organs. Hitzenberger and Tuchfeld (1930) were able to demonstrate considerable accumulation of lactic acid in the blood in their case, and this would contribute to the supply of hydrogen ions not subject to removal by the lungs, and therefore causing over-ventilation.

The final picture of cardio-respiratory failure during exertion requires no analysis, and indicates merely the encroachment on the last physiological reserve of the ventricular muscle.

Effects of Oxygen Inhalation.

In Case II, this was conducted by means of the Benedict portable type of closed circuit metabolism apparatus. It was found that after 10 minutes, the respirations increase in amplitude until the distress caused the subject to discard the mouthpiece and pant for air. It was assumed that this was the result of oxygenation of more than the usual amount of haemoglobin, causing interference with the removal of carbon dioxide from the tissues by reducing the carbamino-haemoglobin formation on which most of the CO_2 carriage depended, and at first intervals of only 10 minutes were used for treatment. The colour of the patient changed rapidly, and after four breathing periods of 10 minutes, the arterial oxygen saturation rose to 90 p.c. The explanation may be that the presence of so much carbamino-haemoglobin in the corpuscles diminishes the rate of oxygenation at the

ordinary partial pressures, much in the manner of carboxy-haemoglobin. Campbell, Hunt and Poulton reported three cases with 69, 77, and 84 p.c. oxygen saturation of the arterial blood, and found it possible to increase the saturation to 91 p.c. by oxygen inhalation. Peters and Van Slyke (1931) suggest that this indicates incomplete oxygenation of that fraction of blood which does pass through the pulmonary circuit. It is difficult to find an organic cause for this, and the explanation tentatively suggested might explain the relatively rapid effect of the respiration of pure oxygen. Column 3 of Table 1 shows the condition of the blood in Case II, after 6 months of oxygen therapy. The gas was inspired for a total period of 30 minutes daily.

In Case I, the erythrocyte count of 7·10 million provides sufficient base to carry 17·5 vols. p.c. more carbon dioxide than normal, whilst in Case II, on one occasion when a count of 10 million was reached, a carbon dioxide carriage of 60 vols. p.c. at 35 mm. pressure, as indicated in the figure (3), would be provided for by the extra corpuscular buffering. This would mean a virtual pH of 7·56 at that pressure.

The partial failure of the "Bohr Effect" will not, in the presence of so much reduced haemoglobin, interfere with carbon dioxide removal; whilst the increased circulation time probably compensates for the slower evolution of oxygen from the oxyhaemoglobin. It does not therefore constitute the disadvantage attributed to it by Hitzenberger (1930). By suitably combining rest and small daily oxygen inhalations from a closed circuit apparatus fitted with carbon dioxide absorber, it has been possible to retard the ill-effect of rising viscosity, and secure longer intervals of moderate well-being, in Case II, over a period of 7 years.

SUMMARY.

The haemoglobin increase in anoxaemia due to congenital cardiac defect may actually depress the oxygenation of the tissues owing to increased circulation time.

The increase in haemoglobin is as much a response to demand for buffering of hydrogen ions, as to demand for oxygen, since owing to anoxia of the nervous mechanism of respiratory control, there is disturbance of the $\frac{\text{acid}}{\text{base}}$ equilibrium, by over-ventilation of the lungs.

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